IMP-3 Complex Protects its Target mRNAs from Argonaute/GW182/miRNA-Dependent Translational Repression

Evgeny Deforzh

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Par

Evgeny DEFORZH

Le complexe IMP3 protège ses ARNm cibles de la répression traductionnelle dépendante de Argonaute/GW182/miRNA

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Composition du Jury :

Yegor VASSETZKY	Directeur de recherche (Institut Gustave Roussy, 94805 Villejuif)	Président
Daniel GAUTHERET	Professor (Univérsité Paris-Sud, 91405 Orsay)	Rapporteur
Slimane AIT-SI-ALI	Directeur de recherche (Univérsité Paris Diderot, 75205 Paris)	Rapporteur
Yegor VASSETZKY	Directeur de recherche (Institut Gustave Roussy, 94805 Villejuif)	Examinateur
Isabelle DUSANTER	Directrice de recherche (Institut Cochin, 75014 Paris)	Examinatrice
Anna POLESSKAYA	Chargée de recherche (Ecole polytechnique, 91128 Palaiseau)	Directeur de thèse

to Vladislav Felicianovich Hodasevich and Kasimir Severinovitch Malevitch.

CONTENTS

ABBREVIATION LIST

3'UTR : 3'Untranslated region
5'UTR : 5'Untranslated region.
AGO : Argonautes
AMPK: AMP-activated protein kinase
ARE: AU-rich Element
RNA Pol II: RNA polymerase II
rRNA : ribosomal RNA
tRNA : Transfer RNA
CCND1 : cyclin D1
CCND3 : cyclin D3
CDK : Cyclin-dependent kinases
CEFs : chicken embryo fibroblasts
EMCV : encephalomyocarditis virus
EMT: epithelial-mesenchymal transition
GFP: Green Fluorescent protein
GW182: Glycine-tryptophan protein of 182 kDa
HMGA2: High Mobility Group AT-hook 2
HNRNPs : Heterogenous Nuclear Ribonucleoproteins
IGF2 : Insulin like growth factor 2
IGF2BP: IGF-2 mRNA Binding protein
IMPs : IGF2 mRNA binding proteins
IRES : Internal Ribosome Entry Site
KD: Knock down
KH domain: K homology domain
KOC: KH domain containing protein overexpressed in cancer
MAPK: mitogen-activated protein kinase
miR: microARN
miRISC: miRNA induced Silencing Complexe
mTOR: mammalian Target of Rapamycin
NES: Nuclear export signal
NLS: Nuclear localization signal
NMD: nonsense mediated decay
nt: nucleotide
ORF: Open Reading Frame
P-bodies: Processing bodies
PABPC: Poly(A) Binding Protein Cytoplasmic
Pan2-Pan3: PAB-specific Ribonuleases 2 and 3
PARN: Deadenylase poly(A) Ribonuclease
pRB: retinoblastoma protein
RBD: RNA binding domain
RBP: RNA Binding Protein
RD: eRMS cell line
RMS: Rhabdomyosarcoma
RRM: RNA recognition motif
RT-qPCR: quantitative Reverse Transcription-Polymerase Chain Reaction)
SG: stress Granules
siRNA: small interfering RNA

Resume

RNA-binding proteins of the IMP family (IGF2 mRNA-binding proteins 1-3) are conserved oncofetal proteins, regulating transport, stability and decay of multiple mRNAs. IMPs are involved in embryonic development and tumorigenesis by controlling cell proliferation, differentiation, migration, polarization and many other important aspects of cell function. IMP-3 is hardly detectable in normal adult tissues, but is overexpressed in many cancers, where it has been reported as a marker of tumor aggressiveness, rapid growth, and bad prognosis for patients. In our research we utilized a rhabdomyosarcoma (RMS) cell line RD, where IMPs were first described as IGF-2 mRNA regulating proteins. We aimed to elucidate the mechanism by which IMP3 regulates the expression of cyclins D1 and D3, thereby contributing to the understanding of oncogenic processes in RMS.

In this study, we show that IMP3 regulates the expression of cyclin D1 and D3 in a significant manner in vivo. We also demonstrate that in the absence of IMP3, the mRNAs of the cyclins are exported to the cytoplasm and associated with polyribosomes, but not translated. IMP3 inhibition does not influence the stability of cyclin mRNAs. We demonstrate that in human cancer cells, IMP3 interacts with multiple RNA-binding proteins, and that a number of these IMP-3 partners impacts on the expression of cyclins D1 and D3. These observations suggest the existence of a regulatory IMP-3 containing RNP complex on the 3'UTR of mRNAs of cyclin D1 and D3. Our results show that an inhibition of two key proteins of RNA-induced silencing complex (RISC) (AGO2 and GW182/TNRC6) rescues the expression of cyclin D1 and D3 proteins, which is significantly decreased in the absence of IMP3 or its protein partners ILF3/NF90 and PTBP1. Therefore, IMP3 and RISC complexes can compete for cyclin mRNAs translational repression/activation. We also identified a number of miRNAs that can be involved in this process, and characterized functionally important regions within 3' UTRs of the cyclins, where the competition between IMP-3 and RISC complexes takes place.

Our results are consistent with the existence of IMP3 - containing multiprotein complex, which is associated with 3'UTRs of the cyclins and regulates their translation by protecting them from miRISC-dependent translational repression.

Résumé

Les protéines se liant à l'ARN de la famille IMP sont les protéines oncofoetales conservées, qui régulent le transport, la stabilité et la traduction de plusieurs ARNm cibles. Les IMPs sont impliqués dans la tumorigenèse et dans le développement embryonnaire par le contrôle de la prolifération cellulaire, la différenciation, la migration, la polarisation et d`autres processus cellulaires. IMP-3 est difficilement détectable dans des tissus adultes normaux, mais il est surexprimé dans les nombreux cancers, où il a été caractérisé comme un marqueur d'agressivité et de la croissance tumorale rapide, ainsi que d'un pronostic défavorable pour les patients. Dans notre étude, nous avons utilisé une lignée cellulaire RD de rhabdomyosarcome (RMS), où IMPs étaient initialement décrits comme des protéines régulatrices de l`ARNm de IGF-2. Nous avons essayé d'élucider le mécanisme par lequel IMP3 régule l'expression des cyclines D1 et D3, contribuant ainsi à la compréhension des processus oncogéniques dans les RMS et autres cancers.

Nous avons montré que IMP3 régule l'expression des cyclines D1 et D3 d'une manière significative in vivo. Nous avons également démontré, qu'en absence de IMP3, les ARNm des cyclines sont exportés vers le cytoplasme et s'associent avec les polyribosomes, mais ne sont pas traduits. En outre, l'inhibition d`IMP3 n'a pas d'influence sur la stabilité des ARNm des cyclines. Nous démontrons que dans des cellules cancéreuses humaines, IMP3 interagit avec plusieurs protéines se liant à l'ARN, et que nombre de ces protéines a un effet sul l'expression des cyclines, ce que suggère l'existence d'un complexe régulateur multiprotéique sur les 3'UTR des cyclines D1 et D3. Nos résultat montrent que l'inhibition de deux protéines clés de RNA-induced silencing complex (RISC) (AGO2 et GW182/TNRC6), rétablit les niveaux d'expression des cyclines D1 et D3, qui ont été considérablement diminués en l'absence d'IMP3 ou de ses partenaires protéiques ILF3/NF90 et PTBP1. Nous concluons que les complexes d`IMP3 et RISC peuvent concourir pour la régulation des ARNm des cyclines. Nous avons également identifié les miARNs qui peuvent être impliqués dans ce processus, ainsi que les domaines fonctionnellement importants dans les 3 'UTR des cyclines, où se passe la competition entre les complexes d'IMP-3 et RISC.

Nos résultats sont compatibles avec l'existence de IMP3 - contenant complexe multiprotéique, qui est associé à 3'UTRs des cyclines et régule leur traduction en les protégeant contre la répression traductionnelle par miRISC.

Introduction

1. The insulin-like growth factor-2 mRNA-binding protein family (IGF2BPs).

1.1. The discovery of IMPs.

The insulin-like growth factor-2 (major fetal growth factor) mRNA-binding protein family comprises three mRNA-binding proteins : IGF2BPs/IMPs 1-3, which, as their name suggests, can bind RNAs and influence their cellular fate : localization, turnover, and translational control.

The nomenclature of the IGF2BP protein family remains confusing. It includes many synonyms: IMP, CRD-BP, VICKZ, ZBP1, Vg1RBP/Vera or KOC. In order to simplify the nomenclature of IMP family members, Dr. Yisraeli proposed the acronym VICKZ. The V is the first identified protein that binds Vg1 mRNA (Vg1RBP or VERA) [Zhang Q et al., 1999]. The I defines IMPs. C comes from the CRD-BP, K corresponds to KOC, Z to ZBP1. The human IMP1 (huIGF2BP1) has two orthologs : CRD-BP (muIGF2BP1) with an almost perfect homology in mice and ZBP1 (chIGF2BP1) with 95% homology in chicken. Moreover, IMP3 (huIGF2BP3) is identical to KOC and has 83% of homology with its ortholog in Xenopus, termed Vg1RBP/Vera. There is no ortholog described for IMP2. The closest to IMP2 is a protein, lacking the N-terminal RRM domain but comprising four KH-domains, that was identified in Drosophila, and is known as Drosophila IGF2BP (dIMP). Thus, multiple studies over the years have referred to these proteins with a variety of names (Fig. 1), and this Introduction will attempt to remain faithful to the original publications with regard to the nomenclature. However, in my own work in human cancer cells, I mostly used the abbreviation « IMP 1-3» or « IMP/IGF2BP 1-3» for these proteins, following the pioneering studies of Nielsen and Christianssen (discussed below).

Evolutionally conserved IGF2BP family members and their orthologs are involved in many important processes during development and cancer, such as cell polarization, migration, changes in morphology, metabolism, proliferation and differentiation. The first described mammalian family member was IGF2BP1, which was initially identified in 1999, when Bernstein and colleagues searched for factors that bind to specific regions of polysome-associated c-myc mRNA and thereby alter its half-life. By UV-cross-linking experiments, IGF2BP1 was detected as a 75-KD polysome-associated protein bound to the 182-nucleotide fragment of c-myc mRNA in a coding region (c-myc-coding region determinant, CRD) and involved in protection of these region of c-myc mRNA from endonuclease attack [Bernstein PL et al., 1992].

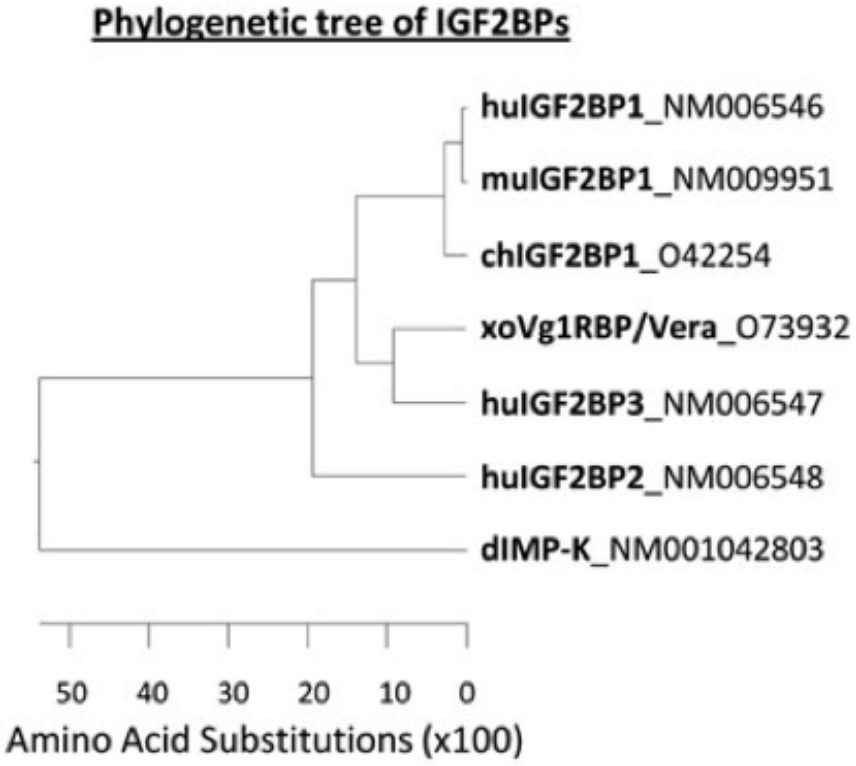


Figure 1. Phylogenetic tree indicating amino acid substitutions of distinct IGF2BP paralogs from different species (*hu* human, *mu* murine, *ch* chicken, *xoXenopus*, *dDrosophila*) [Bell JL et al., 2013].

IGF2BP1 prevents c-myc mRNA degradation and thereby promotes tumor cell proliferation and survival in various cancer contexts [reviewed in Bell JL et al., 2013]. Later on, Ross and Singer have isolated and cloned the 68-KDa ZBP1 (zipcode-binding protein 1, IGF2BP1 ortholog in chicken) that was found to control the subcellular localization of β-actin (ACTB) mRNA in primary fibroblasts and neurons. ZBP1 binds to conserved elements within ACTB mRNA 3`UTR, and notably to a 54-nucleotide element, which has been termed the 'zipcode' [Ross AF et al., 1997]. ZBP1 was proposed to play an essential role in subcellular localization of β-actin mRNA to the sites of active actin polymerization, enhancing neurite outgrowth and axonal guidance [Huttelmaier S et al., 2005].

Human IMP2/IGF2BP2 was first described in 1999 as an insulin-like growth factor II mRNA binding protein, overexpressed in liver cancers [Nielsen J et al., 1999]. Later, this protein, also termed p62, was identified as an auto-antigen in hepatocellular carcinoma [Zhang JY et al., 1999]. Some time later, single nucleotide polymorphisms (SNPs) were found within the

second intron of the human IGF2BP2 gene. IGF2BP2 gene variants with such SNPs were correlated with an elevated risk of developing type 2 diabetes [Christiansen J et al., 2009]. Recently, IGF2BP2 was identified as a positive regulator (enhancer) of cap-independent IGF2 mRNA translational initiation after mTOR-dependent phosphorylation in the linker region connecting the RRM and KH domains. IGF2BP2 was also identified as a modulator of mTOR signaling [Dai N et al., 2011].

Human IGF2BP3, that is highly overexpressed in pancreatic cancer cell lines and in pancreatic cancer tissue as compared to normal pancreas and chronic pancreatitis tissue, was initially termed KOC (KH domain containing protein overexpressed in cancer) [Mueller-Pillasch F et al., 1997]. Since this first identification, high levels of its expression were also found in tissue samples of other human tumors [Findeis-Hosey JJ et al., 2011].

1.2. Structure of IGF2BPs.

In mammals, all three IGF2BP proteins show a high level of amino acid sequence identity (79–84%) and are strikingly similar in order and spacing of domains [reviewed in Bell JL et al., 2013] (Fig. 2).

All three human IGF2BP proteins contain two RNA-recognition motifs (RRMs) in their N-terminal part, four hnRNP-K homology (KH) domains in the C-terminal part, and linker regions between conserved domains. The C-terminal KH domains of the IGF2BPs are essential for RNA-binding and thereby determine the subcellular localization of all three human family members, which is typically characterized by a mainly cytoplasmic, granular distribution [Wachter K et al., 2013]. Recent structural analyses as well as NMR studies of human IGF2BP1 (also termed ZBP1) KH-domains 3 and 4 suggests an anti-parallel pseudo-dimer structure formation, where KH3 and KH4 each specifically recognize the targeted RNA in two appropriately spaced RNA motifs [Chao JA et al., 2010 ; Patel VL et al., 2012]. Forming IGF2BP-RNA complexes in vitro, IGF2BPs can change a conformation of associated transcripts and keep it with surprisingly long half-life (in vitro half-life of such complexes is greater than 2 h). Thus, IGF2BPs play an essential role in the formation of stable protein–RNA complexes. According to in vitro studies, RNA-binding is mainly facilitated by the KH domains, whereas the RRMs promote the stability of protein–RNA complexes and mediate the association with other RBPs [Wachter K et al., 2013 ; Nielsen J et al., 2004]. Despite the high degree of sequence identity in the KH and RRM domains, all three paralogs associate with the IGF2 mRNA but apparently exhibit distinct RNA-binding properties, and show differences in biological functions. These differences can be explained by the highly

variable linker regions [Wachter K et al., 2013].

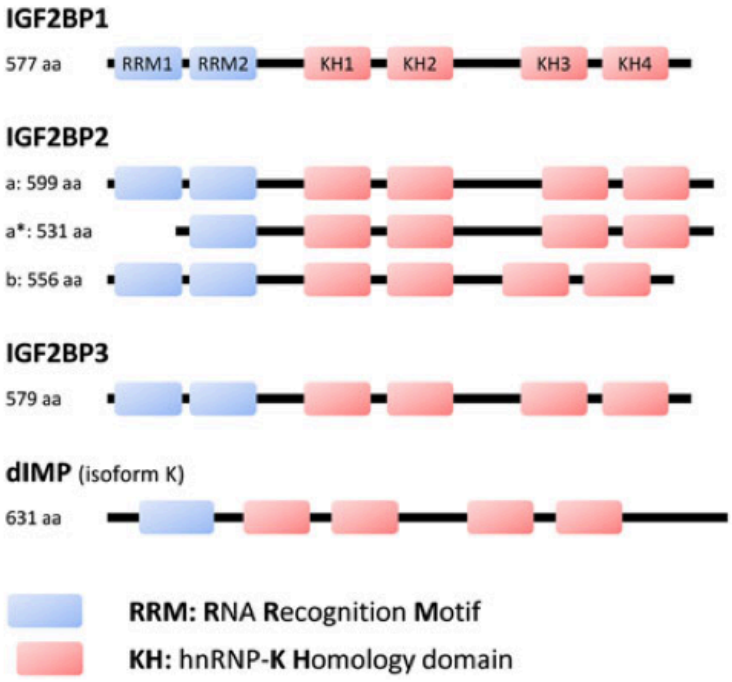


Figure 2. Domain structure of IGF2BPs [Bell JL et al., 2013].

1.3 Expression profile of IMPs.

IGF2BP/IMP family members are highly expressed during the period between zygote and embryo stages [Hansen TV et al., 2004]. A sharp peak in expression occurs around embryonic day 12.5 (E12.5) before a decline in expression towards birth in mice [Hansen TV et al., 2004 ; Hammer NA et al., 2005]. At E12.5, IGF2BPs are highly expressed in the brain, limb buds, muscle and epithelia of many organs in mice. In Xenopus embryo, Vg1RBP/Vera is highly expressed in neural cells. It plays a role in neural crest migration and is required for the migration of cells forming the roof plate of the neural tube [Yaniv K et al., 2003].

IGF2BP1 and IGF2BP3 are highly expressed during embryogenesis, but their expression levels in adult tissues are negligible, with the exception of reproductive tissues [Hammer NA et al., 2005]. In contrast, IGF2BP2 is largely expressed in different adult tissues, except pancreas, with an up-regulated expression in 80-week-old mice [reviewed in : Yaniv K et al., 2002; Yisraeli JK, 2005; Christiansen J et al., 2009]. Huttelmaier group performed a semi-quantitative RT-PCR to analyze the expression of IGF2BPs in various adult tissues in mouse. They confirmed previous reports and showed that IGF2BP1 expression in adult life is almost

absent, with a modest expression observed in the brain, lung and spleen of 16-week-old male mice. IGF2BP3 also demonstrates minor expression levels in the lung, spleen, kidney, and gut of male mice. Consistent with previous studies, largely age-independent expression of IGF2BP2 was observed by Huttelmaier in all adult mouse tissues, except pancreas. Hence, looking at expression patterns of IGF2BP1 and IGF2BP3, we can classify these proteins as 'oncofetal', meaning their almost complete absence in adult tissues, but *de novo* synthesis or upregulation in various tumors and tumor-derived cells [reviewed in : Yaniv K et al., 2002; Yisraeli JK, 2005]. It seems that IGF2BP2 is the only family member to regulate mRNA fate in non-transformed adult tissues, probably with a specific role in metabolic control [reviewed in Christiansen J et al., 2009].

1.4 Control of IGF2BP/IMPs expression at transcriptional level.

The regulation of IMPs expression at transcriptional level is an almost unexplored area. Activation of beta-catenin/TCF4 signaling is implicated in the development of human cancers. The Noubissi group showed that in HEK293 cells, β-catenin (CTNNB1) induces transcription of IGF2BP1 in a TCF4-dependent manner, promoting IGF2BP1-dependent stabilization and translation of BTRC and MYC mRNA [Noubissi FK et al., 2006]. However, this observation remains controversial, since the authors propose that without CTNNB1/TCF4 overexpression, IGF2BP1 is barely detectable in HEK293 cells, whereas multiple other studies indicate that IGF2BP1 is highly abundant in HEK293 cells [Huttelmaier S et al., 2005; Jonson et al., 2007; Mayr C et al., 2009]. Noubissi shows that c-myc enhances IGF2BP1 transcription by binding to its promoter [Noubissi FK et al., 2010], while IGF2BP1 stabilizes the myc mRNA by protecting the transcript from endonucleolytic attack [Lemm I et al., 2002], suggesting a positive feedback loop. IGF2BP1 was also proposed to prevent miR-182 - dependent BTRC degradation, but BTRC promotes the degradation of CTNNB1, suggesting a negative feedback regulation of IGF2BP1. Taken together, these observations provide evidence for CTNNB1/TCF4 and MYC-dependent transcriptional activation and stabilization of IGF2BP1, and support the idea of an oncogenic role of IGF2BP1.

Little is known about transcriptional regulation of other IGF2BPs, and especially, of IGF2BP3. With regard to IMP2/IGF2BP2, Brants JR and colleagues clearly indicate that architectural transcription factor HMGA2 (high mobility group A2) regulates its expression during embryogenesis. This report shows that IGF2BP2 expression, but not that of two other family members, is downregulated in two HMGA2-deficient mouse strains [Brants JR et al., 2004]. HMGA2 binds to the AT-rich region in the first intron with enhancer elements of the

IGF2BP2 gene both in vitro and in vivo [Cleynen I et al., 2007]. Furthermore, they show that a nuclear factor-kappaB (NF-kappaB) cooperates with HMGA2 to positively regulate the transcription of IGF2BP2/IMP-2.

1.5 Control of IGF2BP/IMP family expression at post-transcriptional level.

In addition to transcription, IMPs expression is subjected to a posttranscriptional control. Bartel laboratory observed an interesting phenomenon in cancer cells, where the protein products of oncogenes are sometimes overexpressed without alteration of the proto-oncogene. They found that cancer cell lines often express large amounts of mRNA isoforms with shorter 3' untranslated regions (3'UTRs) [Mayr C and et al., 2009]. Several short isoforms of IMP1 are found in HEK293 cells. These shorter isoforms usually resulted from alternative cleavage and polyadenylation (APA). Authors believe that 3`UTR shortening provide escaping strategy from miRNA-dependent repression, illustrating this hypothesis by the fact that expressing the shorter but not the full-length isoform of the proto-oncogene IMP1 led to oncogenic transformation.

Peter ME's group, with the help of comparative bioinformatics, have identified 12 conserved oncofetal genes regulated by the heterochronic microRNA let-7. These mRNAs include IMP-1/CRD-BP, which was experimentally confirmed to be a direct target of let-7 [Boyerinas B et al., 2008]. Taking into account the tumor suppressive role of the let-7 microRNA family, these data support the view that IGF2BP1 enhances tumor cell aggressiveness. Targeting of dIMP by let-7 has also been shown in Drosophila [Toledano H et al., 2012].

Recent studies indicate that two isoforms of the mRNA-binding protein IGF2BP2 are generated by alternative translational initiation [Le HT et al., 2012]. Huttelmaier group found at least three protein isoforms of IGF2BP2, expressed in several tumor-derived and transformed cells [Bell JL et al., 2013].

Finally, no studies have demonstrated post-transcriptional regulation of IGF2BP3/IMP3.

1.6. Nuclear roles of IGF2BPs.

IGF2BPs are predominately cytoplasmic, usually with an ability to form or to be included within different RNP granules. Their nuclear role remains controversial, but there are reports, where IGF2BPs were shown to form complexes with newly transcribed mRNA in the nucleus. This mRNA-protein association can be essential for subsequent cytoplasmic mRNA localization [Oleynikov Yet al., 2003; Huttelmaier S et al., 2005; Pan F et al., 2007].

A nuclear role for IMP orthologs was also described using Xenopus laevis as a model system. Indeed, a protein called B3.65 was isolated through its ability to bind a cis-regulatory DNA sequence of the TFIIIA gene (transcription factor IIIA), essential for transcription of the 5S rRNA gene. The B3.65 protein has 97% of identity with Vg1 RBP/VERA, ortholog of huIGF2BP3/IMP3. Interestingly, the activation of the TFIIIA transcription corresponds to a nuclear localization of B3.65/Vg1RBP/VERA [Zhang Q et al., 1999].

In the same organism, during spermatogenesis, nuclear accumulation of IMP1 and IMP3 was observed [Zhang Q et al., 1999]. For IMP orthologs in chicken and mouse, two putative NLS signals (Nuclear Localization Signal) were described : a first one between RRM1 and RRM2 domains, and a second one between RRM2 and KH1 domains [Doyle GA et al., 1998; Zhou Z et al., 2002]. However, these two sequences were not experimentally validated to induce nuclear import and it was subsequently suggested that a nuclear localization of IMPs depends on their protein partners [Nielsen FC et al., 2002]. Moreover, a sequence analysis of the IMPs identified two NES (Nuclear Export Signal) within RNA-binding KH domains 2 and 4 [Nielsen J et al., 2003]. In addition, the NES motif within the KH4 domain is was described in dIMP in Drosophila.

Other important observations suggest a role for IMPs in the events of splicing within the nucleus. Indeed, in HeLa cells, IMP3 was found in complex with the spliceosome [Zhou Z et al., 2002]. In addition, IMPs can be associated with nonsense-mediated decay (NMD) factors and exon junction complex factors (EJC). It is probable that IMPs bind their target mRNAs in the nucleus and form RNPs complexes before nuclear export [Huttelmaier S et al., 2005; Nielsen J et al., 2003 ; Oleynikov Yet al., 2003]. Once in the cytoplasm, these mRNPs associate with different macromolecular machines of mRNA translation, transport and/or degradation. Therefore, even though the functional part of the IMP - dependent regulation of RNA fate occurs in the cytoplasm, the initial, critical event of protein-RNA binding must take place in the nucleus (see examples below).

1.7. Cytoplasmic roles of IMPs.

IMPs are mainly cytoplasmic and ‘cage’ their target mRNAs, forming large mobile ribonucleoprotein (RNP) granules, dispersed in the peri-nuclear region and in cellular protrusions. These RNP granules, or ‘locasomes’, are unique RNPs, which differ from the neuronal Staufen and/or fragile X mental retardation protein granules, processing bodies (P-bodies) and stress granules.

Within IMP1 granules, we can find the 40S ribosomal subunit and proteins that bind RNA

and poly(A) tail [Huttelmaier S et al., 2005]. Moreover, IGF2BP granules contain CBP80 and factors belonging to the exon-junction complex. Suprisingly, they lack eIF4E, eIF4G, and 60S ribosomal subunits. This observation suggests that mRNAs in these bodies are translationally repressed or have never been translated.

Cytoplasmic IMP1-containing mRNPs were characterized by a proteomic approach, revealing association of IMP1 with various RNA-binding proteins [Jonson L et al., 2007]. Generally, IMPs interact with RBPs in RNA-dependent manner [Jonson L et al., 2007; Weidensdorfer D et al., 2009]. For example, IMP1 with other RBPs (HNRNPU, SYNCRIP, YBX1, and DHX9) stabilizes c-myc mRNA by associating with coding region instability determinant (CRD). However, some RNA-independent interactions have been identified for some family members. In Xenopus, Vg1RBP/VERA interacts with RBP60/HNRNPI in a RNA-independent manner in the nucleus of oocytes [Lewis RA et al., 2008]. Curiously, in the cytoplasm, this interaction becomes RNA-dependent. This observation suggests that the IMPs containing RNP complexes are dynamic complexes that are revised during nuclear export.

In humans, numerous protein partners and a large number of target RNAs indicate that IMPs are able to form a variety of complexes with many protein-protein and protein-RNA interactions. Furthermore, different IMP-interacting RBPs are involved in mRNA stability control (for example, AUF and HuD1), in the regulation of translation (FMRP) and in the control of mRNA localization (HuD and FMRP). It should also be noted that IMPs interact with many proteins of the cytoskeleton. In Xenopus, for example, Vg1/VERA interacts with microtubules [Elisha Z et al., 1995]. Human and Drosophila IMP1 granules are often found in well-organized rows, suggesting an association with the cytoskeleton [Christiansen et al., 2002].

It is estimated that about 3% of the mRNA transcriptome is found in IMP1 granules [Jonson L et al., 2007]. Most often, these mRNAs are involved in the protein degradation pathway via proteasome, such as those encoding for E3 ubiquitin ligases of the RING finger family (RNF4, RNF26, RNF130 and others). There are also mRNAs encoding the quality control proteins of endoplasmic reticulum and protein secretion.

According to in vitro studies [Nielsen J et al., 2004], IGF2BPs form a stable protein-RNA association that can prevent the premature decay and promiscuous translation of specific target transcripts, for example, CD44, MYC, PTEN or BTRC, supposedly by limiting a release of these transcripts from associated proteins [Lemm I et al., 2002; Kobel M et al., 2007; Stohr N et al., 2012 ; Vikesaa J et al., 2006 ; Noubissi FK et al., 2006]. IGF2BPs seem to be essential for controlling both mRNA localization and translation via directed recruitment

of targeted transcripts to cytoplasmic mRNPs. This stable IGF2BP-mRNAs association (mRNP) promotes 'long-distance' transport of the transcripts and their impermanent storage.

Vg1 RNA-binding protein (Vg1RBP/Vera), an IMP3 ortholog in Xenopus, associates with Vg1 mRNA via specific VLE domain and directs its transport to the vegetal pole of the oocyte during oocyte maturation. During transport, the translation of Vg1 mRNA is inhibited [Deshler JO et al., 1997; Schwartz SP et al., 1992]. The injection of anti-Vg1 antibodies inhibits correct localization of Vg1 mRNAs by 50% [Kwon et al., 2002].

IMP1 ortholog in chicken, the chicken zipcode-binding protein (ZBP-1), participates in the localization of β-actin mRNA to the leading edge of embryonic fibroblasts and neurons.

ZBP1 co-localizes with mRNA of β-actin in the lamellipodia of chicken embryo fibroblasts (CEFs). The localization of β-actin mRNA in the lamellipodia is important to maintain the polarity and cell motility [Farina KL et al., 2003]. With the help of KH3 and 4 domains, which directly bind the zipcode region of β-actin mRNA, ZBP1 promotes the formation of RNPs granules containing β-actin mRNAs [Ross AF et al., 1997]. After the removing of the N-terminal domain of ZBP1, RNP granules can be observed, but they are not accumulated at the extremities of CEFs. This N-terminal domain seems to be important to target the destination of RNP granules. On the other hand, KH3 and KH4 domains also promote association of these granules with microfilaments of actin.

Chicken ZBP1 was also observed in discrete foci in the nucleus, coinciding with the β-actin mRNA transcription sites, indicating that ZBP1 binds to newly synthesized β-actin transcripts. After the nuclear export, ZBP1 can act as a translational regulator of β-actin mRNA in the cytoplasm, preventing premature translation by blocking translational initiation. When ZBP1–RNA complex reaches its destination at the periphery of the cell, Src kinase phosphorylates a key tyrosine residue in ZBP1, required for RNA binding. This event decreases ZBP1 affinity to the mRNA of β-actin [Huttelmaier S et al., 2005]. The subsequent release of mRNA promotes translation. These findings confirm that ZBP1 plays a major role in temporal and spatial control of β-actin mRNA translation.

In neuroblastoma cells (NG 108-15), ZBP1 binds mRNA of β-actin co-transcriptionally in the nucleus [Singer RH, 2003]. Once in the cytoplasm, ZBP1 does not only ensure the transport of the mRNA of β-actin, but also appears to prevent premature translation. This hypothesis is confirmed by the ability of ZBP1 to inhibit translation initiation of β-actin mRNA in vitro. Indeed, ZBP1 prevents the formation of the 80S ribosomal complex.

Translation, therefore, starts when the mRNA reaches its destination at the periphery of the cell, after phosphorylation of ZBP1 by Src protein kinase [Huttelmaier S et al., 2005].
In Drosophila, dIMP binds oskar mRNA at the IBE sites (UUUAY). At the oocyte posterior, IMP colocalizes with oskar mRNA in IBEs-dependent manner. After the mutation of subset or all IBE sites, oskar mRNA doesn't translate and localize to the posterior pole of oocyte [Munro TP et al., 2006]. However, in the absence of dIMP, oskar mRNA translates normally and still have a 'posterior' mRNA localization. These observations suggest that activation of oskar translation may occur under the influence of another unknown IBE-binding factor, redundant to dIMP in its action. Alternatively, the unknown IBE-binding factor can exclusively activate oskar translation, and dIMP can compete with its binding by masking IBE sites [Munro TP et al., 2006].

1.8. IMPs in the control of mRNA translation and stability.

By 'Caging' their target transcripts in mRNPs, IGF2BPs increase their stability and inhibit their translation [Lemm I et al., 2002; Noubissi FK et al., 2006]. Indeed, IMP1 and IMP3 stabilize CD44 mRNA [Vikesaa J et al., 2006], which has five IMPs binding sites within its 3 'UTR. After HeLa cells treatment with actinomycin D (a transcriptional inhibitor), the absence of IMP1 or IMP3 causes a significant decrease in the half-life of CD44 mRNA.
Similarly, IMP1 seems to protect the mRNAs encoding c-myc and MDR1 from endonucleolytic cleavage and degradation [Lemm I et al., 2002; Sparanese D et al., 2007]. When IMP1 binds c-myc mRNA in the coding region, it specifically recognizes with high affinity the CRD site (Coding Region Determinant instability), which is susceptible to be attacked by a polysome-associated endoribonuclease [Sparanese D et al., 2007 ; Bergstrom K et al., 2006]. Indeed, during the translation, the movement of ribosomes on the c-myc mRNA is slowed down at the beginning of CRD site which contains rare codons. The mechanism by which IMP1 stabilizes c-myc mRNA remains poorly understood, but it is possible that IMP1 prevents the degradation of c-myc by impeding its co-translational cleavage in a way of protection of c-myc transcripts in mRNP complex [Weidensdorfer D et al., 2009]. IMP1 works in a complex with four RBPs, which interact with each other in a CRD-dependent manner. Each of these proteins is essential for the stabilization of c-myc through the CRD. Indeed, a knock-down (KD) of IMP3 or of one of its four protein partners by siRNA leads to a two-fold decrease in c-myc mRNA half-life. Moreover, an association between IMP1 and c-myc inhibits its rapid co-translational degradation. Interestingly, the expression of IMP1 in malignant tumors correlates with high expression of c-myc mRNA [Kobel M et al., 2007].

Interestingly, a study of various lineages of human cancers suggests that IMP1 interferes with miRNA-dependent degradation of BTRC (beta-transducin repeat Containing E3 ubiquitin ligase protein) [Elcheva I et al., 2009]. miR-183 interacts with the coding region of BTRC. Curiously, BTRC also has a binding site (CRD) within the same region, which promotes stability by recruiting IMP1. This suggests that IMP1/CRD-BP competes with miR-183 for coding region binding, and that CRD-BP association prevents mRNA silencing by the miRNA pathway. Cytoplasmic IMP1 and 3 granules, locasomes, or « safe houses » also protect HMGA2 from AGO2/let-7 mediated repression, by impacting on the stability of HMGA2 mRNA [Nielsen FC et al., 2014].

The most prominent and frequently studied target of IGF2BPs is the IGF2 mRNA. The IGF-II gene generates overlapping primary transcripts that result in multiple mRNAs with identical coding regions and 3′ UTRs, but distinct 5′ UTRs, referred to as leader 1, 2, 3, 4. In K562 cells (Leukemia cell line), IMP3 is capable to bind the 5`UTRs of IGF2 leader 3 and 4 and promote the translation of the mRNA IGF2 leader 3 [Liao B et al., 2005 ; Suvasini R et al., 2011]. The inhibition of IMP3 by RNAi results in the reduction of endogenous protein level of IGF2 without decrease in mRNA leader 3 and 4 levels. In addition, the KD of IMP3 causes a dose-dependent translational repression of IGF-II leader 3–luciferase reporter construct. These results suggest that IMP3 promotes the translation of IGF2 leader 3. In contrast to IMP3, IMP1, being associated with 5`UTR, directs a repression of IGF-2 leader 3 at the translational initiation level [Nielsen J et al., 1999]. It was recently shown that phosphorylation of IGF2BP2 in the N-terminal linker region between RRM2 and KH1, by mTORC1, promotes the association with the 5`UTR of IGF2 leader 3, resulting in elevated IGF2 protein synthesis [Dai N et al., 2011].

In conclusion, despite the high degree of sequence identity, the IGF2Bps can regulate the cellular fate of the same RNA in a different manner. These observations suggest that IGF2BPs are not absolutely redundant in their functions. Moreover, having a common set of targets, IGF2BPs associate with variable transcripts. For example, unlike IMP1, IMP3 does not bind to the c-myc and β-actin mRNA [Liao B et al., 2005, Suvasini R et al., 2011].

However, how to explain why some IGF2BP targets, being ‘caged’ in cytoplasmic mRNPs, are regulated in a opposite way ? At least two mechanisms can be proposed [Bell J et al., 2013]. On the one hand, the mRNA fate can be determined by the exact protein composition of various cytoplasmic regulatory mRNPs. On the other hand, the cis-determinants of regulated transcripts can exclusively determine the final mRNA fate. IGF2Bps, in this latter

case, can control the release of regulated transcripts from cytoplasmic mRNPs with different functions.

1.9. Post-translational modifications of IMPs influence their roles in the cell.

Phosphorylation-dependent modulation of the RNA-binding activity of RBPs is one of the essential mechanism regulating the cytoplasmic fate of mRNP-associated transcripts [Rudel S et al., 2011; Ostareck-Lederer et al., 2002]. These cytoplasmic signaling events modulate the release of mRNAs from IGF2BP granules, promoting their translation or decay.

Phosphorylation of IGF2BP1 and Xenopus Vg1RBP (IGF2BP3) by the SRC-kinase (IGF2BP1) or MAPKs (IGF2BP3) regulates growth cone guidance [Huttelmaier S et al., 2005 ; Leung KM et al., 2006 ; Yao J et al., 2006]. Phosphorylation of VgRBP/Vera by MAPKs promotes the release of the Vg1 mRNA from the vegetal cortex of Xenopus oocytes.

In particular, it is suspected that IMP1 phosphorylation between KH2 and KH3 is responsible for the dissociation of β-actin from mRNP complex with a subsequent activation of its translation [Noubissi FK et al., 2006]. Moreover, IMP1 and IMP2 phosphorylation by mTORC1 in the linker region between RRM and KH domains, promotes its association with the 5 'UTR of the IGF2 mRNA and a subsequent translation [Dai N et al., 2011]. For IGF2BP3, phosphorylation at a homologous residue was also reported, but the functional significance of this observation is not clear [Dai N et al., 2013]. Post-translational modifications of IMPs seem to modulate their role as regulators of cytoplasmic fate of specific transcripts.

1.10. IMP target sites in RNAs.

In order to determine the sequences within target mRNAs, bound by IGF2BPs, scientists use a variety of methods. RIP-ChIP was the first technique which allowed to study RNPs at large scale. RNA-binding protein precipitation with the antibodies was followed by the analysis of coimmunoprecipitated mRNAs by microarrays [Tenenbaum SA et al., 2000]. Later, in order to improve the detection power of this technology, researchers applied deep sequencing methods instead of microarrays (RIP-Seq). These two technologies identified single RBP associated with hundreds of mRNAs. The introduction of a UV cross-linking step, that ‘froze’ RNA-protein interactions, allowed the identification of RBP targets in living cells. This method was called cross-linking and immunoprecipitation (CLIP). CLIP is followed by sequencing or microarray analysis. Then, it was upgraded and gave rise to photoactivatable ribonucleoside enhanced CLIP (PAR-CLIP) and individual nucleotide resolution CLIP

(iCLIP). PAR-CLIP is consistent of the incorporation of photoreactive ribonucleoside analogs (4-thiouridine (4-SU) and 6-thioguanosine (6-SG)) into nascent RNA transcripts in living cells. UV irradiation of the cells induces cross-linking of photoreactive nucleoside-labeled cellular RNAs with interacting RBPs. Subsequent immunoprecipitation of the RBP is followed by the isolation of cross-linked and co-immunoprecipitated RNA. The isolated RNAs are converted into a cDNA library and deep sequenced using high-throughput sequencing technology. PAR-CLIP was used for identification of the transcriptome-wide binding sites for microRNA-containing ribonucleoprotein complexes (including AGO and TNRC6/GW182 proteins). iCLIP technique uses UV light to covalently bind RNA molecules with located in close proximity proteins. After, there is a purification of linked protein-RNA complexes step, including immunoprecipitation with SDS-PAGE and membrane transfer. The radiolabeled protein-RNA complexes are then cut out from the membrane, and treated with proteinase, resulting in one or two amino acids at the RNA cross-link site. The RNA is then reverse transcribed. Because reverse transcription stops prematurely at the cross-link site, we can identify binding site locations with high accuracy, which allows us to identify the sequence motifs for known RBPs and their locations within the transcript in vivo [Ule J et al., 2003; Hafner M et al., 2010 ; Konig J et al. 2010, reviewed in Konig J et al., 2012]. Finally, SEQRS can be used to refine sequence motifs. This method is composed of in vitro selection, high-throughput sequencing of RNA and SSL (sequence-specificity landscapes). It is extremely useful to analyze the specificity of RBPs, and to study the modulation of RBP-binding specificity by its protein partners and co-factors [Campbell ZT et al., 2012].

Many RBPs bind structured elements within mRNA. This binding is based on the recognition of the mRNA structures rather than on nucleotide sequence identification. PARS and SHAPE-Seq are recently developed genome-wide methods for RNA secondary structure identification. Using a wide range of described methods, we can discover novel RBPs, novel yet-unknown RNA-binding domains, create a catalog of protein–mRNA contact sites (‘mRNA interactome’), for each cell line [Castello A et al., 2012 ; Baltz AG et al., 2012].

Many attempts were made to define the target sites of the IMPs, using a variety of methods. The Singer group searched for the IMP1/ZBP1 binding site within the 3 'UTR of β-actin mRNA. With a help of band-shift mobility assays, UV cross-linking, and affinity purification experiments, they found that ZBP1 recognizes the conserved ACACCC sequence within 54-nucleotide zipcode [Ross AF et al., 1997]. The same procedure used to show that Vg1RBP/VERA recognizes UUUCUA and UUCAC repeats in the 366-nucleotide VgLE

from the 3′ UTR of Vg1 mRNA in Xenopus [Havin L et al., 1998 ; Deshler JO et al., 1998]. In humans, IMPs recognize the UCACGUUCAC sequence of mRNA leader 3 of IGF2. That was demonstrated by mobility-shift assay [Nielsen J et al., 1999]. Drosophila dIMP binding to UUUAY motifs of oskar 3`UTR was demonstrated by SELEX method [Munro TP et al., 2006].

Further studies suggest that IMPs directly bind to secondary structures within mRNA via their KH3 and KH4 domains [Chao JA et al., 2010]. Indeed, as was shown by EMSA studies, that the sequence of the 'zipcode' ACACCC is not sufficient for IMP1 recognition. In 2009, Robert Singer lab identified two 'zipcodes' sequences: 5`element (5`-GGACU-3`), bound by KH4, and 3`element (5`-ACA-3`), bound by KH3. A mutation in one of these sequences results in the loss of mRNA binding by KH3 and KH4, indicating that these domains recognize a bipartite sequence within 'zipcode'. It is interesting that a space between the two sequences, bound by individual KH3 and KH4, is consistent with the structure of domains KH3 and KH4 of IMP1, obtained by X-ray crystallography. However, KH domains do not bind mRNA with the same affinity as the whole protein [Nielsen J et al., 2004].

In 2010, the Tuschl lab used the PAR-CLIP technique to identify the mRNA sequences recognized by the IMPs. They utilized HEK293 cells that stably express a tagged form of IMP1, 2 and 3 (FLAG-HA-IMP). 4-thiouridine-labeled transcripts were crosslinked to RBPs and partially digested by RNase. RNA-protein complexes were immunopurified and bound RNA was size-fractionated by SDS-PAGE. RNA molecules were eluted, associated RBPs were digested by proteinase K. RNA were converted into a cDNA library and deep sequenced. With PAR-CLIP method, authors showed that IMPs have a slight preference for the 3'UTR in comparison to coding sequence. They also determined that IMPs have more than 1,000 targets and recognize their targets through a CAUH pattern (H = A, C or U) [Hafner M et al., 2010].

Therefore, it is clear that despite the high degree of conservation between IMP orthologs, and high IMPs homology within the same species, particularly within canonical RNA-binding motifs, no generally accepted IGF2BP target site has been clearly defined so far. It also hard to explain why the three IGF2BPs often demonstrate different affinities for the same mRNA, and why they have a wide variety of non-overlapping RNA targets [Yisraeli JK, 2005]. The study of interactions between IMPs and their targets is very complex in the 'absence' of a unique consensus sequence or pattern especially as the mRNA secondary structure also appears to play an important role in the recognition by IMPs [Nielsen FC et al., 2002]. In addition, IMPs are capable to form homodimers and heterodimers on their target mRNAs.

This dimerization further complicates the target identification but may explain the high stability of IMPs mRNPs-complex [Nielsen FC et al., 2002].

1.11. Role of IGF2BP during development.

The role of IMP proteins during development has been studied with the use of over-expression and knock-down/knock-out experiments.

Constitutive reexpression of KOC, the ortholog of IMP3, in transgenic mice under the control of metallothionine promoter, promoting its expression in the stomach, pancreas, liver and kidney, causes no any visible alterations, and mice demonstrate normal growth and behavior [Wagner M et al., 2003]. However, this overexpression leads to discrete changes in the micro-architecture of the pancreas.

In Drosophila, dIMP overexpression disrupts the dorsal/ventral polarity [Thomas Hays, 2008 ; Geng C et al., 2006]. In Xenopus, a down-regulation of Vg1RBP, an ortholog of IGF2BP family, was performed, using 'antisense oligonucleotides', called morpholinos (AMO). This led to many morphological defects : loss of lateral pigments, reduced or absent dorsal fin, impaired lens formation, abnormal morphology of the head (Yaniv et al., 2003). These defects are explained by inability of neural crest cells and neural tube roof plate cells to migrate and reach their destinations, although their differentiation markers are normal. After an injection of sense Vg1 RBP RNA, insensitive to AMO-directed degradation, a normal embryonic phenotype is restored with high efficiency. These experiments show the essential role of Vg1RBP/VERA in cell migration during Xenopus embryogenesis.

ZBP1 loss-of-function inhibits the correct localization of β-actin mRNA, which is required for dendritic filopodia formation, and causes defects in synaptogenesis in rat neuronal cells [Tiruchinapalli DM et al., 2003, Eom T et al., 2003]. Interestingly, an increase in the density of dendritic filopodia and the number of synapses has been observed in mouse neuronal cells, overexpressing constructs containing the cis-acting, $3'$ -untranslated region signal (zipcode sequence) of β-actin mRNA. Moreover, ZBP1 regulates axon guidance in mouse, modulating local translation of β-actin with the help of neitrin-1 [Welshhans K et al., 2011].

In Drosophila, dIMP is a part of large RNase-sensitive complex that is present in the polarized developing oocytes and neurons. Loss of function of dIMP causes significant defects in synaptic terminal growth [Boylan KL et al., 2008]. Taken together, these observations determinate IGF2BPs as key regulatory factors of neuronal development that modulate outgrowth and cell migration of neurons.

IGF2BP1 is the only paralog for which knockout mice have been described. IMP1 - / - mouse is characterized by severely reduced viability (50% of them were alive 3 days after birth), dwarfism (40% smaller than wild-type) and impaired gut development [Hansen TV et al., 2004]. It was suspected that smaller sized organs can be caused by hypoplasia. PCNA, a marker of proliferating cells, was reduced and a marker of apoptosis (TUNEL staining) was not significantly increased compared to wildtype mice. According to this report, IGF2BP1, apparently, is a key factor of embryonal development and perinatal survival, promoting cell growth and differentiation, presumably through IGF2 regulation [Nielsen J et al., 1999].
For the other IMPs no *in vivo* models were generated.
Interestingly, there is substantial evidence, showing IGF2BPs to modulate cell adhesion, the formation of invadopodia, and intrinsic cell polarization. Notably, IGF2BP2 could modulate cell adhesion. Recent reports, including work from our laboratory, suggest that IGF2BP2 controls the expression of critical component of the glomerular basement membrane LAMB2 [Schaeffer V et al., 2012]. Moreover, IMP2 regulates PINCH-2, an important mediator of cell adhesion and motility ; IMP2 controls microtubule (MT) stability via MURF-3 regulation [Boudoukha S et al., 2010]. The inhibition of IMP1 and IMP3 in Hela cells affects cell adhesion and formation of invadopodia via the regulation of CD44 mRNA [Vikesaa J et al., 2006].

1.12. IMPs and cancer.

Expression of IGF2BP proteins has been observed in various cancers; however, most of these reports refer to IMP1 an IMP3, confirming their role as 'oncofetal' proteins. IMP1 and IMP3 are mainly expressed during embryogenesis, but become de novo synthesized in various malignancies : cancer of colon, liver, kidney, pancreas, female reproductive tissues and others. Although IGF2BP2 expression was detected in liposarcoma, liver cancer, and endometrial adenocarcinomas [Cleynen I et al., 2007 ; Zhang L et al., 2011 ; Lu M et al., 2001], its role in cancer remains less clear.
Pro-oncogenic role of IGF2BP1 was demonstrated by only one in vivo study, using a transgenic mouse model. In this study, the expression of IGF2BP1 was induced under the control of whey acidic promoter (WAP) upon lactation in mammary epithelial cells of adult female mice [Tessier CR et al., 2004]. Within the period when IGF2BP1 was highly expressed (the first 60 weeks), the incidence of mammary tumors was up to 95 %. In the period of lower relative expression of IGF2BP1, the incidence was around 60 %. The levels

of IGF2 and H19 mRNAs were significantly increased in mammary tissues, presumably promoting tumor growth.

IGF2BPs are involved in the regulation of classic oncogenes, like MYC and KRAS. There are many observations suggesting a positive role of IGF2BP1 in MYC expression in various tumors in vitro (in mammary carcinomas [Ioannidis P et al., 2005]; in ovarian carcinomas [Kobel M et al., 2007]; in colorectal carcinomas [Mongroo PS et al., 2011]).

As described above, IGF2BP1 regulates the stability of MYC mRNA by preventing its endonucleolytic cleavage within coding region [Weidensdorfer D et al., 2009 ; Lemm I et al., 2002 ; Sparanese D et al., 2007 ; Bergstrom K et al., 2006]. MYC as well as KRAS are well known targets of let-7 miRNA family. IGF2BP1 can also interfere with let-7 microRNA, which targets the 3-UTRs of both transcripts. IGF2BP1 enhances KRAS expression. This fact is presented in only one study, but the mechanism remains elusive [Mongroo PS et al., 2011].

The role of IMP1 in metastatic process is poorly understood due to lack of correlation between in vitro and in vivo studies. The question about pro-metastatic role of IMP1 remains open. Numerous observations suggest that IMP1 promotes cell migration [Vikesaa J et al., 2006]. There is also a positive correlation between IMP1 expression and metastasis of colorectal carcinomas [Vainer G et al., 2008]. Moreover, the expression levels of IMP1 is in direct correlation with the metastatic process in rat adenocarcinoma (MTLn3). There is a strong evidence for a pro-metastatic role of IGF2BP1 in vivo, since transgenic expression of the protein in mice induced primary breast cancer lesions as well as metastasis [Tessier CR et al., 2004]. However, in human breast cancer cell lines T47D and MDA231, it was shown that IMP1 inhibits the invasive capacity of the cells by controlling the localized expression of various mRNAs involved in cell adhesion and motility. Thus, IMP1 is important for localized expression of E-cadherin and β -actin [Gu W et al., 2012].

IMP3, in contrast to IMP1, is not well studied *in vivo*, but recent observations implicate IMP3 as an important marker of tumorigenesis [Kapoor S, 2008 ; Findeis-Hosey JJ et al., 2010] and a general indicator of malignancy [Wagner et al., 2003]. Over 40 studies have shown elevated expression levels of IMP3 in different types of cancer [Bell JL et al., 2013]. The role of IGF2BP3 in the regulation of IGF2 mRNA translation remains contradictory, but recent in *vitro* studies in different human cancers indicate that elevated expression levels of IGF2BP3 might promote IGF2 translation, enhancing tumor growth [Suvasini R et al., 2011]. It has been shown that IMP3 promotes cell growth, proliferation, and the resistance to ionic radiation via IGF2 regulation [Liao B et al., 2011]. Moreover, it is interesting to note that

there is a correlation between IMP3 and increased in vitro invasive and metastatic ability of gliomas [Vikesaa J et al., 2006 ; Suvasini R et al., 2011 ; Samanta S et al., 2012]. Regulation of CD44, one of the validated targets of IMP3, may explain this correlation. Indeed, IMP1 and IMP3 promote the formation of invadopodia, stabilizing CD44 mRNA [Vikesaa J et al., 2006].

Our group has shown that IMP3, with the help of HNRNPM, enters the nucleus and binds the mRNAs of cyclins D1, D3, and G1, positively regulating their expression, and promoting tumor cell proliferation [Rivera Vargas T et al., 2013]. IGF2BP3 was identified as one of the most severely upregulated RBPs in HCCs [Chen CL et al., 2013 ; Jeng YM et al., 2008 ; Gutschner T et al., 2014]. Recently it was shown, that IGF2BP3 protects HMGA2 (the architectural transcription factor) from let-7 family miRNA-dependent repression [Jonson L et al., 2014]. Consistently, IGF2BP3 expression was strongly correlated with an enhancement of HMGA2 expression in hepatocellular carcinomas (HCCs). IGF2BP3 was proposed to enhance tumor cell aggressiveness through HMGA2 upregulation in lung carcinoma cells, as well [Kumar MS et al., 2014]. The oncogenic role of IGF2BP3 is also supported by an in vitro study, where it stabilizes the ABCG2 mRNA, resulting in enhancement of chemo-resistance of breast cancer-derived cells [Samanta S et al., 2013]. Via stabilization of CD44, MMP9 and PDPN mRNAs, IGF2BP3 promotes the invasive potential of tumor cells. IMP1 and IMP3 are also involved in resistance to chemotherapy and radiotherapy treatments [Boyerinas B et al., 2012].

Based on the above-mentioned reports, we can conclude, that IGF2BP in cancer can be involved in regulation of growth, migration/metastasis, survival, chemo-resistance of cancer cells, via the control of subcellular trafficking, translational regulation and stability of their RNA targets.

1.13. Regulation of cyclin D1 and D3 expression.

Cyclins, key coordinators of cell cycle, are highly regulated at all levels (Fig.3). In mammals, many metabolic and mitogenic signals are capable to influence the cell cycle. Wnts, ErbBs, JAKs and STATs pathways are known to activate transcription of CCND1 and CCND3. In addition to promoting transcription and translation, mitogen signals promote the assembly of cyclins with their catalytic subunits CDK4 and CDK6, and a translocation of these complexes to the nucleus [Sherr CJ et al., 1999].

Cyclins D1 and D3 can be regulated on the level of transcription. E2F1 binds to the cyclin D3 promoter in vivo and activates it [Ma Y et al., 2003]. E1AF transcription factor, that plays an

important metastatic role, upregulates Cyclin D3 transcription, increasing cell cycle progression [Jianhai Jiang et al., 2007]. The transition from G0 to S is controlled by Ras [Mulcahy LS et al., 1985] which stimulates transcription of CCND1 [Albanese C et al., 1995]. A study of the proliferating NIH3T3 cell line shows that CCND1 is weakly expressed in S phase. During S/G2 progressing, CCND1 is induced by Ras and reaches the peak of its expression. This high level expression is maintained before the next G1 phase [Hitomi M et al., 1999]. Indeed, the level of CCND1 in G2 is assured by post-transcriptional mechanisms [Guo Y et al., 2002]. In cell culture, the absence of growth factors reduces CCND1 protein synthesis with insignificant changes on its mRNA levels [Brewer JW et al., 2000 ; Mettouchi A et al., 2001 ; Pervin S et al., 2001]. This post-transcriptional regulation of CCND1 remains necessary for cell cycle control.

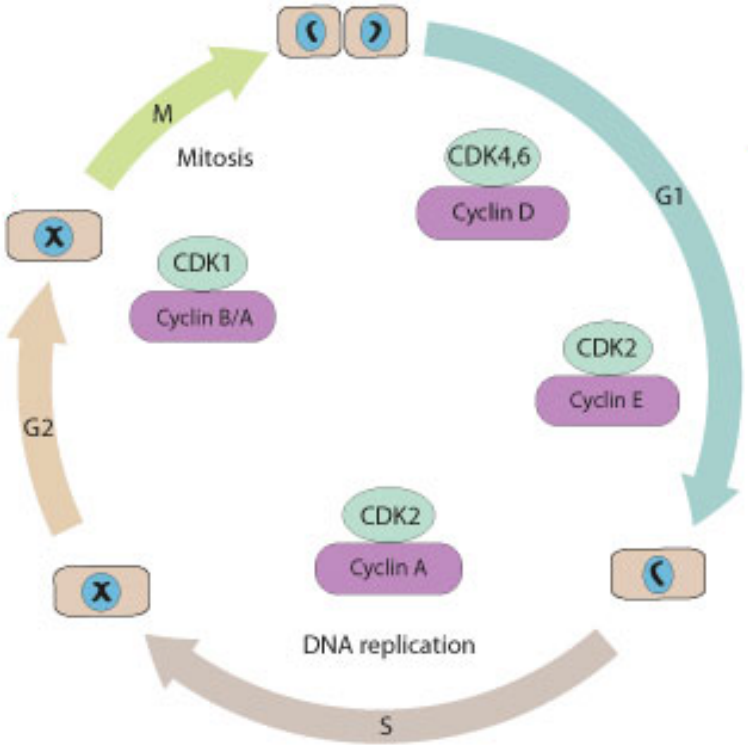


Figure 3. Cell cycle transitions are driven by activities of cyclin/cyclin-dependent kinase (Cdk) complexes [from Bioinformatics Resource Portal].

The CCND1 expression can also be regulated by alternative splicing, resulting in generation of two isoforms : D1a and D1b [Alao JP, 2007]. D1b transcript produces a protein lacking the

PEST domain, suggesting its constitutively nuclear localization and activation [Lu F et al., 2003]. The expression of the cyclins with their rapidly changing levels throughout the cell cycle, is regulated not only at the level of gene transcription, but also by proteolytic degradation [Hershko A, 1997 ; Lania L et al., 1999]. $p38^{SAPK2}$ has been shown to phosphorylate cyclin D1 on threonine residue 286 (T286) and induce its proteasomal degradation [Alao JP, 2007]. Cyclin D3 is degraded via proteasome, through a specific site phosphorylation (Thr-283) by serine-threonine kinases [Casanovas O et al., 2004]. In addition to all these and other regulatory mechanisms, the recent years have seen an increasing number of studies of translational regulation of the cyclins. As these proteins have an extremely short half-life (30-45 min), and their mRNAs are much more stable and abundantly expressed, translational regulation coupled to protein degradation provides flexible and dynamic mechanisms that control the cyclins' protein levels.

CCND1 can be regulated by miRNAs. In human breast tumors, a negative correlation between the levels of miR-17-5p/20a cluster and cyclin D1 was observed [Yu Z et al., 2008]. Interestingly, cyclin D1 can bind the promoter of miR-17/92 cluster and up-regulate its transcription. In turn, miR-17/20 limits the proliferative function of cyclin D1 [Zuoren Yuet al., 2008]. This estimated feedback loop possibly allows the cell to maintain the required level of CCND1. Cyclin D1 can also be regulated by miR-193b in melanoma [Chen J et al., 2010].

A negative correlation between miR-138 and CCND3 mRNA expression was observed in HCC tissues [Huang B et al., 2015]. MicroRNA-503 was reported to downregulate cyclin D3, which results in inhibition of G1/S transition in hepatocellular carcinoma [Xiao F et al., 2013].

An interesting study reports that a cis-acting element, located at the 5' UTR of mammalian CCND3, forms a stable G-quadruplex structure and represses the translation of a reporter gene and the CCND3 gene in human cell lines [Weng HY et al., 2012].

Indeed, post-transcriptional regulation events are particularly important for the control of mRNA amounts, that must be translated rapidly, like the mRNAs involved in cell cycle or inflammatory responses [Mitchell P et al., 2000 ; Gao M et al., 2001].

2. RNA interference. siRNA, miRNA.

The following part of literature review presents an up-to-date point of view on the mechanisms of inhibition of gene expression, transposable elements and viruses by various types of short non-coding RNAs. Observations focus on the data obtained in C. elegans, Drosophila and mammals, which are the principal models in the majority of RNAi studies.

2.1. General characteristics of RNA interference.

The phenomenon of RNA interference (RNAi) is a key mechanism of suppression of gene expression, as well as of genomic repetitive elements and of viral nucleic acids, by means of homologous short RNAs. The detection of RNAi phenomenon in experiments with nematodes [Fire A et al., 1998] revealed a new layer of regulatory processes that are involved in the regulation of gene expression in the majority of eukaryotes. According to some estimates, up to the half of human genes may be regulated by RNAi [Bartel DP, 2009], and without an understanding of this process, it is impossible to receive a complete picture of the cell life.

The functioning of repressive effector complexes RISC (RNA induced silencing complex), containing short RNAs guide systems for silencing of complementary targets, lays at the base of RNAi mechanism. Three main classes of repressive short RNA are recognized currently in mammals: siRNA (short interfering RNA), miRNA (microRNA) and piRNA (PIWI interacting RNA) [Rana TM, 2007]. siRNA take part in cell protection against the viruses and transpositions of mobile elements, and in rare cases can regulate the genes expression of an organism [Carthew RW et al., 2009; Ding SW et al., 2007; Golden DE et al., 2008]. miRNA is the widespread regulator of gene expression, probably involved in the regulation of practically every cellular process [Bartel DP, 2009]. piRNA function as part of an evolutionarily conservative system which is directed to the repression of mobile elements in germ cells of Drosophila and vertebrates, and disturbance of their function leads to the transpositional ‘explosion’ and, consequently, to the loss of fertility [Hartig JV et al., 2007].

After the biogenesis, short RNAs incorporate in RISC complex. A key component of this complex is the protein Argonaute (Ago), which specifically binds to a short RNA. Ago protein family includes two subfamilies: Ago and Piwi [Hutvagner G et al., 2008]. siRNA and miRNA are bound by Ago-subfamily proteins. If siRNA or miRNA, as a part of RISC, are fully complementary to the target RNA, Ago produces its endonuclease cleavage (‘slicer activity’ of Ago). In case of partial complementarity, RISC promotes the translational repression or slicer-independent miRNA degradation. In some cases, effector complexes in

the nucleus can bind to the newly formed transcripts and promote heterochromatinisation [Verdel A et al., 2004]. piRNAs are bound by the proteins of Piwi subfamily, and mechanism of repression in this case is not well studied. The existing data show the possibility of degradation of mobile elements, as well as their transcriptional silencing by piRNAs.

2.2. siRNA.

In plants, fungi and animals, siRNA take part in protection against viruses and transposition of mobile elements. They are necessary for the support of the correct structure of the centromere (S.pombe) and are possibly involved in the regulation of gene expression (Drosophila) [Carthew RW et al., 2009; Ding SW et al., 2007]. In the case of some vertebrates and Drosophila, siRNA are involved in the repression of transposons only in somatic tissues, and in germinal tissues this function is promoted by piRNA.

2.2.1. siRNA biogenesis and loading into RISC.

A characteristic feature distinguishing siRNA from miRNA and piRNA is the origin and the structure of their precursors. The siRNA precursor is a long dsRNA, resulting either from a RNA virus infection (in plants and mammals), or generated after bi-directional transcription of transposons (also shown for plants and mammals), from long RNA 'hairpins' and the overlapping mRNA transcripts (Drosophila, mammals), as well as a result of RNA-dependent polymerases activity (nematode, plants, yeast) [Carthew RW et al., 2009; Ding SW et al., 2007; Golden DE et al., 2008]. siRNA is produced after dsRNA cutting with the help of cellular endoribonuclease Dicer (Fig.4). As a result of the dsRNA processing, siRNA duplexes are generated, consisting of two short RNAs with the size of 21-22 nt (in plants - up to 24 nts). Dicer belongs to a RNAseIII family and therefore performs cleavage in a manner specific for this family - each end of the duplex comprises two protrusive unpaired nucleotides at the 3 'end as well as a phosphate at the 5' end.

The resulting siRNA duplexes are included in the effector RISC complex, interacting with key component of this complex, i.e. protein of Argonaute family (Ago). Apart form Ago, the complex RISC, contains a number of additional proteins. The Dicer not only cuts dsRNA, but it is also a component of the intermediate protein complexes, carrying out the loading of the siRNA in RISC. In Drosophila, Dicer2 acts in a complex with a helper protein R2D2, comprising two domains for dsRNA binding (in mammals, a similar function is performed by TRBP) [Chendrimada TP et al., 2005; Liu J et al., 2003]. R2D2 is not involved in dsRNA

cleavage, but plays an important role in siRNA loading to RISC. In active RISC, which can promote silencing, there is only one strand of the siRNA duplex (guide strand). Another strand (passenger strand) is cleaved by Ago protein and in most cases dissociated (slicer Activity of Ago) [Matranga C et al., 2005; Miyoshi K et al., 2005; Rand TA et al., 2005]. The Dicer2 / R2D2 complex determines which one of the duplex strands will be guide.

The thermodynamically stable end of the siRNA duplex binds to R2D2, and a low melting end binds to Dicer2 [Tomari Y et al., 2004b]. The strand which is incorporated in the active RISC and becomes a guide one, has the 3'-end directed to R2D2. This "thermodynamic rule" is confirmed by many experiments [Hutvagner G et al., 2005; Khvorova A et al., 2003; Schwarz SP et al., 2003]. Although siRNA which is loaded in RISC is a double-strand one [Rand TA et al., 2005], the Ago protein binds only the guide strand and does not form a stable contact with passenger strand [Hutvagner G et al., 2008], which allows its excluding during the formation of an active RISC.

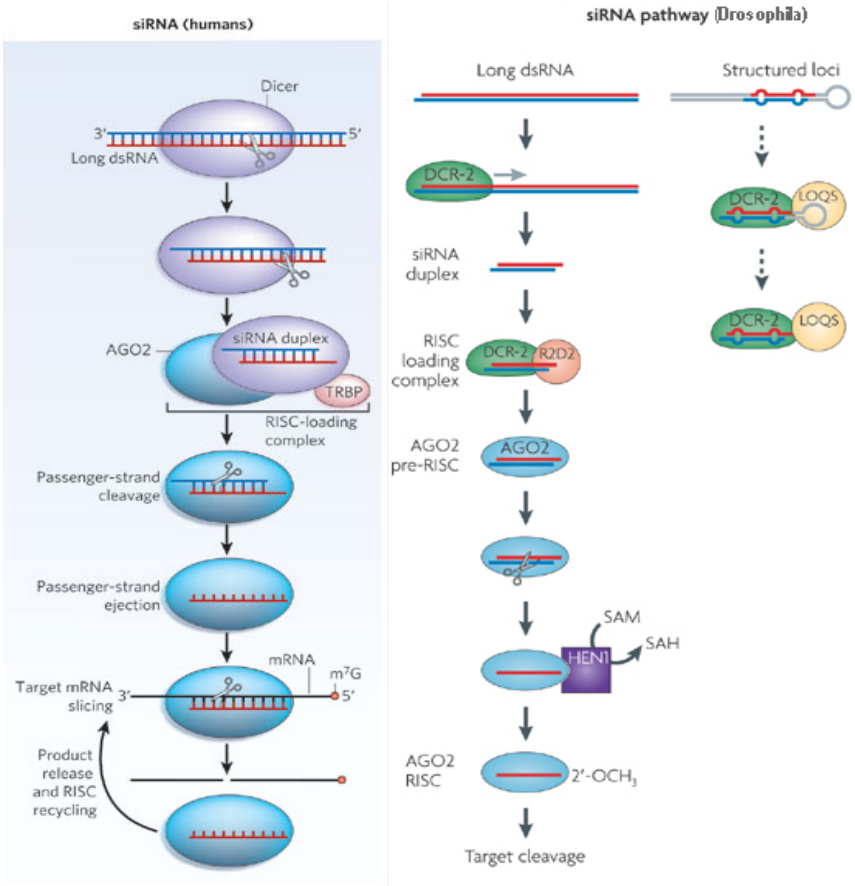


Figure 4. siRNA biogenesis in humans and Drosophila [from Martin Jinek et al., 2009; Ghildiyal et al., 2008] .

2.2.2. Mechanisms of mRNA repression by siRNA.

Degradation of complementary mRNA.

In the effector RISC complex, siRNA is bound by Ago family proteins. Ago proteins contain the conservative PAZ and PIWI domains [Carmell MA et al., 2002]. PAZ domain is composed of two subdomains separated by a pocket, which held two 3' overhanging siRNA nucleotides [Lingel A et al., 2004; Ma JB et al., 2004], interacting with the conservative aromatic and hydrophobic amino acid residues. The phosphorylated 5'-terminal nucleotide of siRNA is anchored inside the pocket of the PIWI domain, which is composed of basic amino acids [Parker JS et al., 2005]. siRNA is positioned on the surface of Ago, like one of the strands of double-stranded A-form RNA. Such arrangement facilitates the scanning and interaction between siRNA and mRNA. 'Out-of spiral' localization of 5' nucleotide, anchored inside the PIWI domain pocket, is in a good agreement with the experimental data and bioinformatics, according to which the pairing of 5' guide strand terminal nucleotide of siRNA or miRNA with mRNA is not necessary for recognition and cleavage of mRNA [Haley B et al., 2004].

PIWI domain contains a structure similar to that detected in RNaseH [Parker JS et al., 2004; Rivas FV et al., 2005; Song JJ et al., 2004]. In RNaseH, this structure is responsible for RNA strand cleavage in RNA-DNA hybrids. RNAseH contains a "catalytic triad" of amino acids AspAspGlu, involved in catalysis. The similar amino acid motif AspAspHis is found in many eukaryotic Ago PIWI domains, including human Ago2. It was shown that AspAspHis motif in PIWI domain of Ago2 is required for the human mRNA cleavage [Liu J et al., 2004; Rivas FV et al., 2005]. Recombinant Drosophila and mammalian Agos, produced in E.coli, can catalyze the cleavage of mRNA by siRNA without any additional proteins [Liu J et al., 2004; Meister G et al., 2004; Miyoshi K et al., 2005; Rivas FV et al., 2005]. Thus, siRNA directs the cleavage activity of Ago protein to the complementary mRNA (slicer activity of Ago). The cleaved phosphodiester bond is localized between mRNA nucleotides, which are complementary to the tenth and eleventh nucleotides from the 5' end of the siRNA [Haley B et al., 2004]. mRNA cleavage products contain 5' phosphate and 3' hydroxyl [Martinez and Tuschl, 2004]. Cellular exonucleases catalyze the degradation of miRNA fragments [Orban TI et al., 2005].

siRNAs formed by viral dsRNA cutting are completely complementary to the viral RNA. Therefore, these siRNA initiate degradation of viral transcripts. In order to achieve a gene KD, scientists typically use a dsRNA that is fully complementary to the targets and causes its degradation. The question arises, what is the mechanism of mRNA suppression in the case of

partial complementarity between siRNA and mRNA? The answer to this question is important for the determination of non-specific siRNA targets. In the presence of unpaired nucleotides in the middle of a short RNA-target RNA duplex, the catalytic center of Ago can not perform the degradation of the targets [Hutvagner G et al., 2002: Saxena S et al., 2003]. Imperfect complementarity is typical for miRNA – mRNA interaction with a result in mRNA translational repression or slicer-independent degradation.

Some data suggest that siRNA-dependent degradation may affect the translated mRNA targets. In Drosophila, a protein dFMR (component of RISC), interacts with ribosomal proteins L5 and L11 [Caudy AA et al., 2002; Ishizuka A et al., 2002]. dFMR knockdown in cell culture reduces the efficiency of target repression [Caudy AA et al., 2002]. In trypanosomes, siRNA are found in polysomal fractions after sucrose gradient fractionation [Djikeng A et al., 2003]. On the other hand, some evidence suggests that the actual translation process is not required for efficient siRNA-dependent degradation. The inhibition of translation at the initiation level by IRE (iron response element) or at the elongation step using hygromycin B, anisomycin or cycloheximide, does not reduce the efficiency of the target mRNA degradation [Gu S et al., 2005; Zamore PD et al., 2000]. Inhibition of translation initiation even increases the efficiency of mRNA degradation, probably because the active ribosomes movement along the mRNA may interfere with RISC binding [Gu S et al., 2005].

A similar effect is observed in the miRNA-dependent translational repression. It seems that the interaction between translation machinery and miRNA switches it into an "open" conformation, available for RISC binding. According to this interpretation, the masked mRNA in Drosophila oocytes are resistant to miRNA-dependent degradation and start degrading only after unmasking and translational initiation (switching to the "open" state) [Kennerdell JR el al., 2002].

2.3. miRNA.

According to various estimations, miRNA may affect between a third and a half of human genes and, apparently, miRNAs are involved in the regulation of almost every cellular process [Bartel DP, 2009]. miRNA differ from siRNA by a typical structure of RNA precursors. miRNA, in contrast to siRNA, are specifically cut out from 'hairpin' precursors and correspond to one of its strands, in most cases.

2.3.1. Biogenesis of miRNA.

miR coding sequences are located within various genomic contexts. In humans, the majority of canonical miRNA genes are located in introns of noncoding or coding transcripts, but some miRNAs are encoded by exonic regions [Minju Ha et al., 2014 ; Cai X et al., 2004; Lee Y et al., 2004a]. miRNA genes are transcribed by RNA polymerase II, to form a pri-miRNA (primary miRNA) with the size of up to 1000 nucleotides (Fig. 5) [Bartel DP, 2004]. Typical animal pri-miRNA forms a 'hairpin' containing a double-stranded region of 33 nt, a terminal loop and two flanking single-stranded RNA. RNaseIII Drosha in combination with dsRNA-binding proteins Pasha in Drosophila [Denli AM el al., 2004] or a human DGCR8 [Han J et al., 2004] cuts out a 'hairpin' of 65-70 nucleotides in length from pri-miRNA, which is called pre-miRNA (precursor of miRNA). At the moment, the exact mechanism defining the cleavage region in pri-miRNA is not clear. According to the predominant point of view, DGCR8 protein binds to the base of a 'hairpin' due to dsRNA-binding domains and orients the catalytic site of Drosha at a distance of 11 nucleotides from the crossing of single-stranded RNA into double-stranded [Han J et al., 2006]. Pre-miRNA contains the characteristic features of RNAseIII processing, 5 'phosphate and two 3' overhanging nucleotides.

Some of the miRNA precursors, located in introns and called mirtrones, can be processed without Drosha. The intron with such miRNA is cut out using the spliceosome: firstly, a lasso-type structure is formed, then it is linearised and folded in a 'hairpin' resembling a pre-miRNA and containing two 3' overhanging nucleotides and 5' phosphate [Ruby JG et al., 2007a]. Such pre-miRNA may be involved in normal miRNA pathway and provide a mature miRNA. 14 mirtrones were identified in Drosophila and 4 mirtrones in nematode. It is proposed that the first pre-miRNA, arising during evolutional process could be formed using the spliceosome, and only after some time Drosha has been adapted for this purpose.

In Drosophila, exportin-5 provides the transport of pre-miRNA from the nucleus to the cytoplasm [Yi R et al., 2003], where the complex of Dicerl with a dsRNA-binding protein

Loqs cuts the miRNA duplex to the size of 21-23 nt (Fig.5) [Forstemann K et al ., 2005]. Dicer1 recognizes the end of pre-miRNA with two overhanging 3' nucleotides, using its PAZ domain. At the present moment, the role of other proteins in pre-miRNA processing is not well documented. The emerging examples of such proteins are the Drosophila Loqs [Forstemann K et al., 2005; Saito K et al., 2005] and TRBP (TAR RNA binding protein) in humans [Chendrimada TP et al., 2005]. There are known three isoforms of Loqs, resulting from an alternative splicing. Only two of them, containing two canonical and one noncanonical dsRBD, interact with Dicer1 and determine its specificity with regard to pre-miRNA [Saito K et al., 2005].

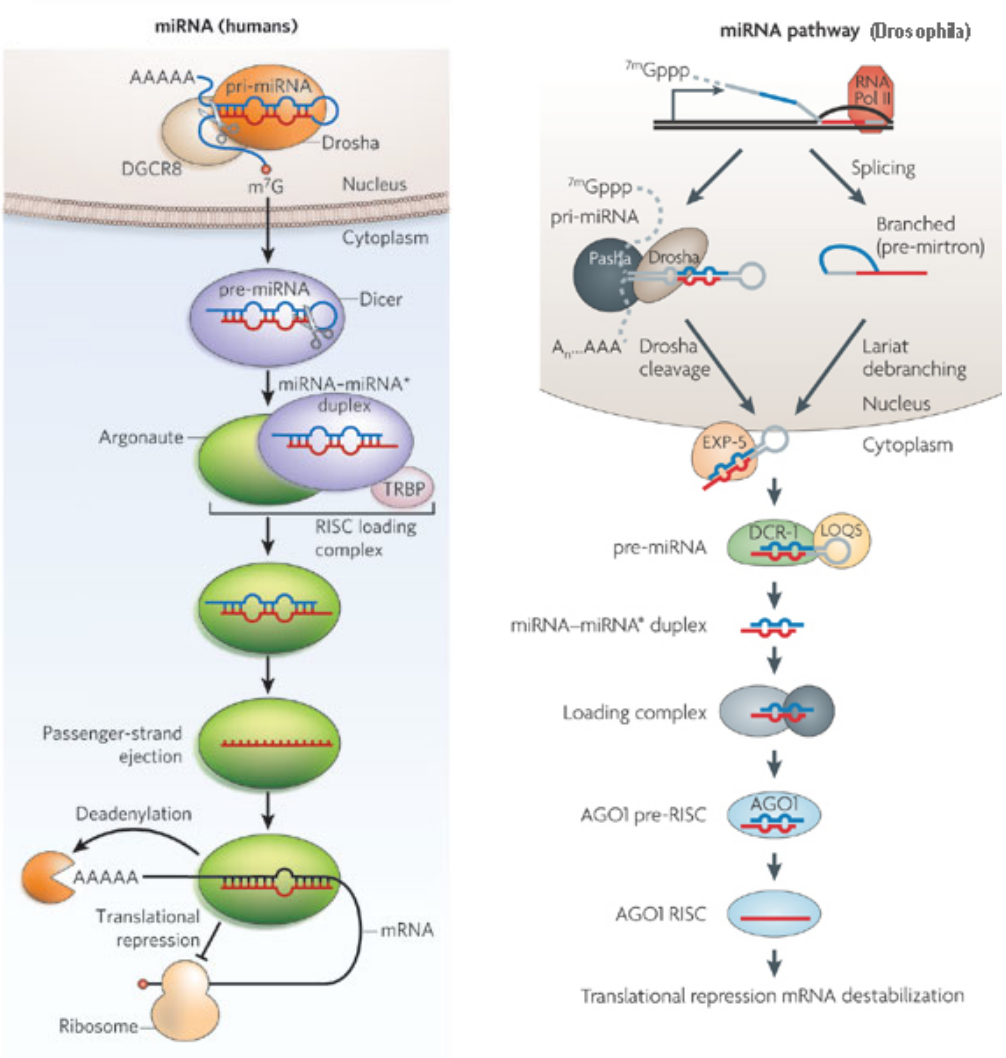


Figure 5. miRNA biogenesis in Humans and Drosophila [from Martin Jinek et al., 2009].

The same Dicer protein can process pre-miRNAs as well as long dsRNAs in human and nematode cells. In other organisms, the existence of different Dicers allows to regulate selectively the processes of siRNA and miRNA formation.

Drosophila Dicer1 is involved in miRNA processing, and Dicer2 is responsible for the formation of siRNA [Lee YS et al., 2004b; Xie Z et al., 2004]. In Arabidopsis thaliana, four genes encoding Dicer-like proteins are found (Dicer like, DCL). DCL3, localized in the nucleus, is involved in cutting long dsRNA to siRNA with the length of 24-26 nt, thus allowing transcriptional retroelement silencing [Hamilton A et al., 2002]. DCL1 is required for the miRNA formation and, apparently, can carry out both stages of pri-miRNA processing without Drosha participation.

Loading of miRNA duplex into the RISC is performed by Dicer together with other proteins. In humans, a Dicer / TRBP / Ago2 complex performs the processing of pre-miRNA and loads the resulting miRNA duplex in Ago2, which leads to the formation of an active RISC [Gregory RI et al., 2005; Maniataki E et al., 2005]. It is shown that the active RISC incorporates guide miRNA, but not passenger miRNA. It means that as in the case of miRNA downloading, Dicer with TRBP can ‘feel’ the more fusible end of the duplex and incorporate miRNA into the RISC according to the "thermodynamic" rule [Gregory RI et al., 2005]. Addition of the miRNA duplex to the RISC results in 10 times more slow degradation of the target than the addition of the equivalent amount of pre-miRNA. This may indicate a more efficient loading of newly miRNA duplexes in Ago2 compared with duplexes that are present in the solution. It seems that the complex Dicer / TRBP / Ago2 quickly provides the cutting of miRNA duplex from pre-miRNA, as well as determining the guide strand, and then loads it into Ago2, working like a highly efficient enzyme complex which catalyzes the consecutive modifications of the substrate. However, recent publications show a regulatory function and activity of passenger strand [Mah SM et al., 2010 ; Okamura K et al., 2008].

In Drosophila, miRNA and siRNA loading into RISC, as well as their biogenesis, is carried out by different systems. A Dicer2 / R2D2 complex binds the short RNA duplexes, where the strands are completely complementary to each other (i.e. siRNA) and loads them into Ago2 [Tomari Y et al., 2007]. Another complex, possibly consisting of Dicerl and Loqs proteins, binds the duplexes with unpaired nucleotides (this is the predominant form of miRNA duplexes) and loads them into Agol. Participation of different Dicer and Ago proteins in miRNA and siRNA metabolism in Drosophila allows to avoid the competition between these pathways, which could lead, for example, after a viral infection with subsequent appearance

of an excess of siRNA, to the disruption of the miRNA-dependent regulation of gene expression.

2.3.2. miRNA interaction with mRNA.

2.3.2.1. 5'- miRNA region plays a major role in the recognition of the mRNA target.

The bioinformatics tools are widely used in searching for miRNA targets. There are many programs created for this purpose, such as TargetScan, PicTar, Miranda, miRBase Targets, PITA Top, mirWIP [Betel D et al., 2008; Friedman RC et al., 2009; Gaidatzis D et al., 2007; Griffiths-Jones S et al., 2008; Hammell M et al., 2008; Kertesz M et al., 2007; Lall S et al., 2006; Ruby JG et al., 2007b]. These programs are used to predict the miRNA targets, which can be subsequently experimentally validated by analyzing the transcriptome (oligonucleotide microarray) or proteome (for example, quantitative mass spectrometry). As a result, new patterns of target recognition are included in subsequent versions. This systematic approach allows to identify the main requirements that are necessary for interaction between miRNA and their targets.

Searching programs impose two basic requirements for the target sites within miRNA: these sites should have complementarity with "seed" miRNA region and should be evolutionally conserved. The seed region consists of nt 2-7 from the 5 'end of miRNA. This area is the most conserved part of animal miRNA [Lim LP et al., 2003]. In some experiments, it is shown that nucleotide changes in the seed region strongly disrupt miRNA-dependent regulation [Brennecke J et al., 2005; Doench JG et al., 2003; Kloosterman WP et al., 2004; Lai EC et al., 2005; Wang B et al., 2006]. Pairing of 6-nucleotide miRNA seed region with target site is necessary, but not sufficient for miRISC binding. The searching algorithm, which gives the largest number of confirmed targets after experimental verification, requires conservation of one and / or two nucleotides in target site, adjacent to the 6 nucleotides (complementary to seed). It is necessary that the nucleotide, which is located opposite to the 1st nt in miRNA at the target site, is an adenine (it does not matter what nucleotide is the first one in miRNA in this case) and / or the nucleotide, which is opposite to 8th nt in miRNA will form a complementary pair with him. Thus, for each miRNA target site within mRNA, programs search for any of the two 7-nucleotides sites, which correspond to the above conditions.

In simplified form, the searching of target sites for miRNA can be divided into three stages. 1) for each miRNA program, two 7- nucleotides target sites are determined; 2) In the available genome sequences of different species, the orthologous 3 ' UTRs are identified; 3) Within these 3 'UTR 7 -nucleotides target sites are searched. The result is a hypothetic target sites. If

there is a small number of genomic sequences, the criterion of conservation is the presence of 7-nucleotide site in all genomes. With more available quantity of genomic sequences, search programs start to include poorly conserved target sites. This is determined by individual settings which differ from one program to another.

For a highly conserved miRNA, this approach reveals a significant number of mRNA targets. Even after subtracting poorly conserved sites, for each family of miRNA (in the miRNA family all the elements contain identical nucleotides 2-8) hundreds of targets usually could be found. For example, mammalian miRNAs which are conserved among the vertebrates, have approximately 300 mRNA targets for a family [Friedman RC et al., 2009]. It is expected that more than a half of the human protein coding genes have the conserved target sites in 3'UTR [Friedman RC et al., 2009].

Experimental data confirm the ability of one miRNA to inhibit the expression of a large number of mRNA targets. According to microarray data, miRNA transfection in HeLa cells causes changes in the expression of hundreds of mRNAs [Lim LP et al., 2005]. Many of these mRNAs contain miRNA seed region complementary sites, which indicates that the effect of reducing of the mRNA quantity can be a direct consequence of miRNA - dependent repression. miRNAs shift the gene expression pattern of HeLa cells towards the tissue which normally expresses this miRNA, indicating that the observed regulation may occur in the organism.

2.3.2.2. The role of the miRNA 3 'end in target recognition.

In theory, additional pairing of the target site with the 3' region of miRNA could increase the affinity between miRISC and mRNA and thereby enhance the silencing. However, experimental data show that additional pairing only slightly increases the efficiency of miRNA-dependent silencing [Grimson A et al., 2007] and in most cases does not play a significant role in the target recognition. In accordance with its small role in the target recognition, additional pairing is found out only in a small percentage of miRNA-target pairs. For example, in Drosophila, in the majority of target sites, additional pairing is found out with a frequency corresponding to the frequency of random occurrence [Brermecke J et al., 2005]. In mammals, 5% of target miRNAs have additional pairing with their target sites [Friedman RC et al., 2009].

One of the functions of additional pairing may be the compensation of single-nucleotide non-complementarity in seed region, which is found out in pairs of let-7 miRNA - lin-41 mRNA in nematode and miR196 - Hoxb8 mRNA in mammals (so-called compensatory pairing)

[Vella MC et al., 2004; Yekta S et al., 2004]. Other members of the family of let-7 - miR-48, miR-84 and miR-241 have the same seed region but different 3 'UTR [Lau NC et al., 2001; Lim LP et al., 2003] and are expressed in the earlier stages of nematode development than let-7 [Abbott AL et al., 2005; Reinhart BJ et al., 2000]. It is assumed that the absence of compensatory pairing in these miRNA prevents the premature mRNA repression of lin-41, which could disrupt the normal development of the nematode. In general, the compensatory pairing is found out only in very few cases [Brennecke J et al., 2005; Friedman RC et al., 2009; Lewis RC et al., 2005].

2.3.2.3. Non-conserved target sites of miRNA.

The functional significance of miRNA target sites is often evaluated by their evolutionary conservation. At the same time, the number of non-conserved 7 nt miRNA target sites exceeds the number of conserved sites about 10 times [Bartel DP, 2009]. Experimental validation of multiple non-conserved sites using the reporter constructs showed an efficient miRNA-dependent repression of such constructs [Farh KK et al., 2005]. However, in the organism, the majority of mRNAs which contain non-conserved sites, is not subjected to repression, as they are expressed in tissues where the complementary miRNA is absent [Farh KK et al., 2005]. This phenomenon can be explained by the "selective avoidance"-evolutionary pressure for highly expressed tissue-specific mRNAs to lose the target sites for the miRNAs present in the same tissue. In addition, in tissues and organs where a given miRNA is not expressed, there may occur random non-conserved complementary sites which will not be functionally important [Farh KK et al., 2005; Stark A et al., 2005]. Some mRNA with non-conserved sites are not a subject of selective avoidance, i.e. they are expressed in the same tissues as the complementary miRNA, and may represent newly established targets.

2.3.2.4. The influence of the mRNA context for the efficiency of miRNA-dependent silencing.

It is shown that the same miRNA target sites can be repressed in some mRNAs and be insensitive to the repression in others [Brennecke J et al., 2005; Farh KK et al., 2005; Giraldez AJ et al., 2006], which indicates the influence of the surrounding sequences on the site efficiency. Currently, several features of the mRNA context of miRNA target sites were shown to increase their efficiency.

First of all, an efficient site must be located in the 3 'UTR, with a distance more than 15 nucleotides from the stop codon. The sites located in the 5 'UTR and ORF (Open Reading

Frame) can cause the repression [Kloosterman WP et al., 2004; Lytle JR et al., 2007], but such repression is much less efficient than for sites in the 3 'UTR [Grimson A et al., 2007]. Bioinformatics and genome-wide experimental analyzes indicate that some part of miRNA-mediated silencing is directed to ORF [Baek D et al., 2008; Easow G et al., 2007; Farh KK et al., 2005; Grimson A et al. 2007; Lewis BP et al., 2005; Lim LP et al., 2005; Stark A et al., 2007], but the number of sites in the 3 'UTR is ten times more. Apparently, miRISC can be removed from mRNA by the movement of ribosome through the 5 'UTR and ORF [Bartel DP, 2004], so the sites in 3 'UTR appear to be more efficient. Since the ribosome during translation termination interacts with the first 15 nucleotides in the 3 'UTR, the sites located in this area are also inefficient [Grimson A et al., 2007].

Second, target sites in the 3 'UTR must not be made inaccessible by secondary structures of RNA. The most efficient are the sites at the ends of the 3 'UTR, since, as it is assumed, there is higher possibility for the forming of secondary structures in the middle of a 3' UTR [Grimson A et al., 2007]. Even more important is the nucleotide composition near the site. It is shown that the sites surrounded by the sequences with high AU content are more efficient [Grimson A et al., 2007]. The reason for this lies in the fact that the potential secondary structure formed by AU sequences is less stable and therefore more accessible to miRISC, than the structure which consists of GC nucleotides.

Third, in a small number of cases, the cooperativity of miRNA effects is detected. If the target sites in a mRNA are located at the distance less than 40 but more than 8 nucleotides, then the summary effect is greater than the sum of the effects of two independent sites [Grimson A et al., 2007; Saetrom P et al., 2007]. In analogy with the transcription factors, it can be assumed that the cooperativity of miRISCs action may lead to repression that is sensitive to small changes in the level of miRNAs. If the complementary sites are located at a greater distance, then the combined effect is the sum of the effects of each site. The above-listed observations of the influence of site location in miRNA to the repression efficiency are included in the number of prediction algorithms for miRNA targets (TargetScan, PITA Thor mirWIP) [Friedman RC et al., 2009; Hammell M et al., 2008; Kertesz M et al., 2007].

2.3.2.5. Limitations of existing ideas about the recognition of miRNA targets.

Despite the significant success in understanding of the miRNA-target recognition mechanisms, at the moment there remain a number of non-resolved issues in this area. First, some functionally efficient sites do not fit the generally accepted rules of target search. For example, the experimentally validated miR-125 sites in the 3 'UTR of LIN28 mRNA in

humans contain 3’ adenosine residues that contribute to the function of the site, without being complementary to the target site in miR-125 [Wu L et al., 2005]. In HeLa cells, miR-16 causes the degradation of TNF-α mRNA, though it contains only partially complementary miR-16 target sites [Jing Q et al., 2005]. It is assumed that the TTR protein (tristetraprolin), which is necessary for miR-16-dependent silencing and which interacts with the mRNA target site and miRISC, is providing such a non-canonical interaction.

Second, the influence of the mRNA context on the efficiency of target site has not been sufficiently taken into account yet. The action of a given miRNA at identical target sites contained within different mRNAs differs in its repression intensity, and only 60% of this variability can be explained on the basis of modern ideas about the influence of context on repression [Grimson A et al., 2007].

Third, the mRNA is a target of many regulatory systems in the cell, which may affect the miRNA-dependent silencing. Some cases of such influence have been discovered. For example, mRNA can avoid miRNA-dependent silencing by alternative processing and polyadenylation, when the target sites are removed from it. In mice, this phenomenon is discovered in various proliferating cells, where the 3 'UTRs of multiple mRNAs are shorter than in non-proliferating cells. Such short 3 'UTRs have in average two times less conserved miRNA target sites than their long isoforms [Sandberg R et al., 2008]. In addition, a mRNA may be protected from silencing by RNA-binding regulatory proteins.

Fourth, the vast majority of the studies of the mechanisms of miRNA targets recognition are performed using highly expressed miRNA. The low-level miRNAs may require special conditions for binding to the targets. The rare miRNAs may involve mechanisms which increase the affinity of the interaction with target mRNA, i.e. they may use miRISC-associated mRNA-binding proteins or form a strong 3'-pairing. There is no doubt that in future, more interactions of miRNA pathways with other regulatory pathways of gene expression will be found, thus highlighting the exact place of miRNA-dependent repression in the overall network of intracellular regulation.

2.3.3. The mechanisms of miRNA-dependent translational repression.

miRNA, as well as siRNA, can be completely complementary to a site in mRNA that causes cleavage of the phosphodiester bond in mRNA, performed by Piwi domain of Ago (slicer activity). In animals, the majority of miRNAs are partially complementary to the sites within mRNAs. If the mRNA nucleotides, between which the cleavable phosphodiester bond is situated, do not form a complementary pair with the miRNA, the catalytic center in Piwi

domain can not perform the cleavage [Hutvagner G, 2002; Saxena S et al., 2003]. In this case, the miRISC binding to mRNA causes its translational repression and / or degradation by slicer-independent mechanisms. While it is sufficient to have only one complementary mRNA site for the effective degradation with Ago, an effective translational repression and slicer-independent degradation usually requires several partially complementary sites, typically located in the 3 'UTR. In some cases, instead of mRNA repression, miRNA may cause the activation of translation [Vasudevan S et al., 2007; Vasudevan S et al., 2007] by mechanisms that will be discussed below.

2.3.3.1. Slicer-dependent degradation of mRNA.

As in the case of siRNA, when a miRNA is fully complementary to the target mRNA, the catalytic site in Piwi domain of Ago cleaves the phosphodiester bond between mRNA nucleotides, which are complementary to nucleotides 10 and 11 in miRNA. This mechanism of repression is widespread in plants, but in animals it occurs rarely [Bartel DP, 2004].

2.3.3.2. Suppression of translation initiation.

If there is no complementarity between nucleotides 10-11 within miRNA and the corresponding nucleotides in mRNA, then miRISC activates a translational inhibition. Currently, the majority of studies concentrate on two possible mechanisms of translational suppression via miRNA. One series of studies indicates a suppression of translational initiation [Bhattacharyya SN et al., 2006; Humphreys DT et al., 2005; Mathonnet G et al., 2007; Pillai RS et al., 2005; Wakiyama M et al., 2007; Wang B et al., 2006], and other groups show that repression happens at post-initiation step [Nottrott S et al., 2006; Olsen PH et al., 1999; Petersen CP et al., 2006; Seggerson K et al., 2002]. In the experiments described below (sometimes they are very similar), the authors have somehow obtained differing data, implicating either the initiation or the post-initiation stage of translation as principal mechanisms of repression. The attempts of many authors to combine these mechanisms in a common regulatory pathway are not yet successful. The working hypothesis for the moment is that the translation of any mRNA can be inhibited by different mechanisms, and the choice between them is determined by specific proteins associated with the mRNA, or by the RISC complex composition.

The data in favor of miRNA-dependent repression of translation initiation.

In cell culture, exogenous target mRNA, synthesized in vitro, leaves the polysomal fractions of sucrose gradient as a result of miRNA-dependent repression [Pillai RS et al., 2005]. This indicates that miRNA interferes with ribosomes binding and inhibits translation initiation.

Several studies have shown that the reporter mRNAs which use the alternative translation initiation mechanism, such as viral IRES (Internal Ribosome Entry Site), are not subject to miRNA-dependent silencing, which indicates that repression is directed on cap-dependent initiation machinery. IRES are highly structured RNA sequences that are able to initiate translation in the absence of cap structure [Filbin ME et al., 2009]. In [Humphreys DT et al., 2005], a culture of mammalian cells was transfected with in vitro synthesized bicistronic mRNA, where the second cistron translation was directed by the IRES of CrPV (cricket paralysis virus) or EMCV (encephalomiocarditis virus). It was found that the same reporter mRNA was resistant to miRNA-dependent repression when containing the CrPV IRES, and

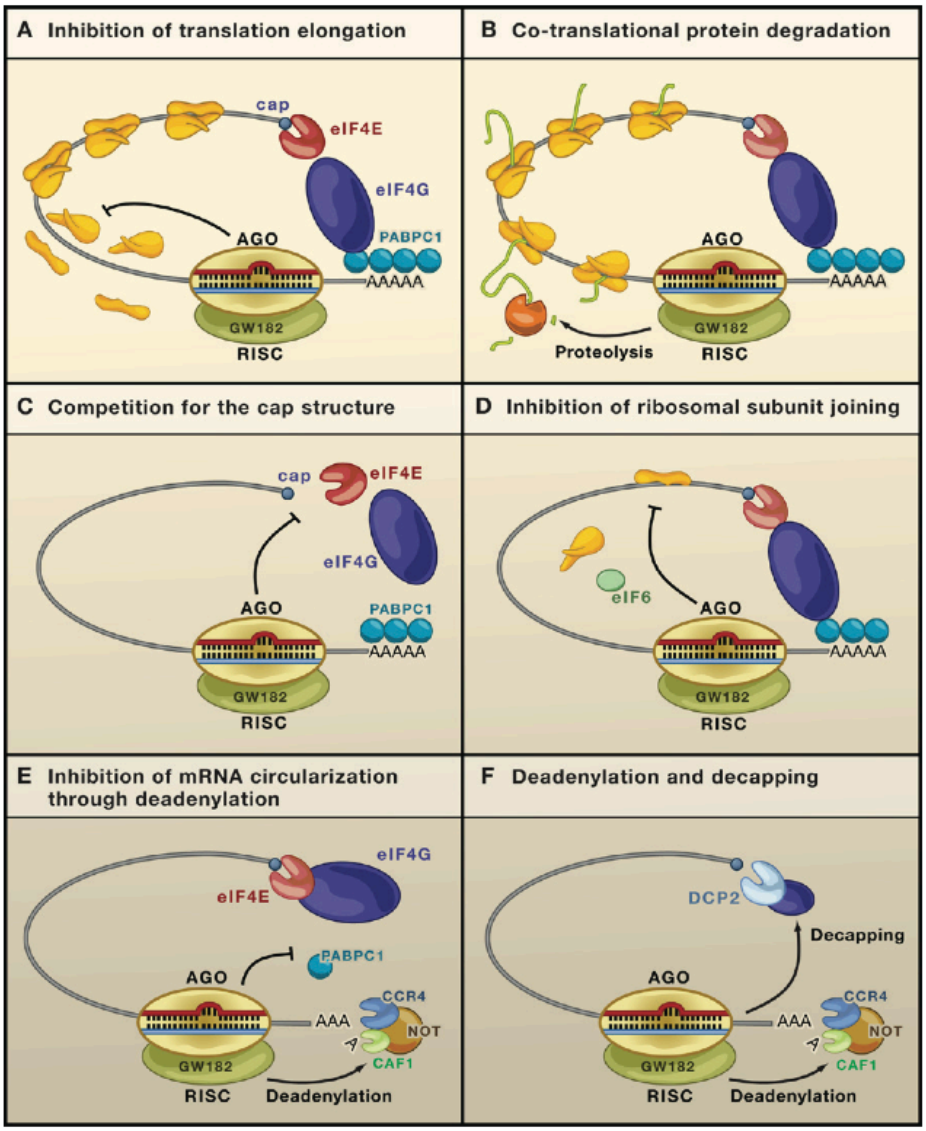


Figure 6. Mechanisms of miRNA-mediated gene silencing [Eulalio and al., 2008a].

was only partially repressed when containing the IRES of EMCV. In a complementary study, using slightly differing methodology, cells were transfected with in vitro-synthesized mRNA containing the IRES of EMCV or HCV (hepatitis C virus) [Pillai RS et al., 2005]. The mRNAs with both IRES were completely resistant to repression using miRNA let-7a. These studies suggested that only cap-dependent, but not IRES-dependent translation is sensitive to miRNA-dependent repression, probably via the initiation factor eIF4E, and that miRISC may disrupt the binding between the initiation complex and the capped mRNA.

The m7Gppp Cap structure cotranscriptionally hinges at the 5 'end of the mRNA in the nucleus and is required for efficient translation of the mRNA in the cytoplasm [Spirin A., 2000]. Cap is bound by the translation initiation factor eIF4E, which is a component of the

eIF4F complex, participating in the small subunit ribosomal recruitment to the 5 'end. In some cases, m7Gppp cap replacement by a nonfunctional ApppN cap as well as IRES element introduction in 5 'UTR (discussed above) can suppress the miRNA-dependent translational repression [Humphreys DT et al., 2005; Mathonnet G et al., 2007; Pillai RS et al., 2005; Wakiyama M et al., 2007; Wang B et al., 2006]. In humans, the protein Ago2 contains a motif similar to the cap-binding motif of eIF4E [Kiriakidou M et al., 2007]. Two conserved tryptophans of eIF4E 'enfold' both sides of methylated guanine base of the cap, forming a stacking interactions. Ago2 has two phenylalanine in a positions equivalent to tryptophans. Ago2 binds m7GTP on the surface of sepharose beads , and the cap-analogue (m7GpppG) competitively inhibits this binding [Kiriakidou M et al., 2007]. Replacement of one or both of phenylalanines by valine disrupts the m7GTP binding and as a consequence, disrupts the Ago2 repressor effect too. It is expected, that after miRISC binding on mRNA, Ago can bind a cap structure, disrupting the eIF4F complex binding to mRNA (Fig. 6C).

Unlike the situation in mammals, the Drosophila Ago proteins do not bind directly to the cap [Eulalio A et al., 2008b; Iwasaki S et al., 2009]. In Drosophila, the protein Ago1 is the main protein involved in miRNA-dependent repression, but in the cases where miRNA duplex has a complete complementarity, Ago2 may participate in silencing too [Forstemann K et al., 2007; Tomari Y et al., 2007]. Ago1 and Ago2 in Drosophila suppress the translation by a variety of mechanisms. Ago1 promotes a mRNA deadenylation, and Ago2 breaks the interaction between eIF4E and eIF4G, which are the members of the initiation complex eIF4F, thereby not allowing the small subunit of the ribosome to bind to target mRNA [Iwasaki S et al., 2009].

2.3.3.3. miRISC may disrupt ribosome recruitment by attracting eIF6 factor.

EIF6 initiation factor binds to the 60S ribosomal subunit and prevents the assembly of the whole 80S ribosome [Ceci M et al., 2003]. In humans, eIF6 was detected in the complex comprising RISC proteins, particularly Ago2, and 60S ribosomal subunits [Chendrimada TP et al., 2007]. The knockdown of eIF6 in human cells or in nematode releases a miRNA-dependent repression of both reporter and endogenous mRNAs [Chendrimada TP et al., 2007]. It is assumed that miRISC may recruit eIF6 to the ribosomes assembly site on mRNA and inhibit the assembly of the whole ribosome (Fig.6D). However, in the Drosophila S2 cell culture, the eIF6 knockdown does not disrupt the miRNA-dependent repression [Eulalio A et al., 2008b]. Thus, eIF6 may participate in miRNA-dependent silencing in mammals, but it is not required for the silencing in Drosophila.

2.3.3.4. miRISC may cause deadenylation of mRNA, thereby disturbing its circularization and therefore reducing the efficiency of translational initiation.

Poly(A) sequence increases the translation of mRNAs [Spirin A., 2000]. PABP (poly(A) binding protein) binds a poly(A) sequence of mRNA and interacts with a translation initiation factor eIF4G, which is a cap-recognizing eIF4F complex component [Gingras AC el al., 1999]. This leads to the formation of the "ring" structure of mRNA, detected by the electron microscopy [Spirin, A., 2000]. The interaction of PABP and eIF4G enhances the translation in Xenopus oocytes [Wakiyama M et al., 2000]. miRNA can cause mRNA deadenylation [Giraldez AJ et al., 2006; Wakiyama M et al., 2007; Wu B et al., 2006] and thus reduce the translation efficiency (Fig. 6E) [Wakiyama M et al., 2007]. In Drosophila, miRISC, which contains protein Ago l, causes the deadenylation of target mRNA [Iwasaki S et al., 2009]. On the other hand, mRNA without poly(A) can also be subject to miRNA-dependent repression [Eulalio A et al., 2009b; Iwasaki S et al., 2009; Wu B et al., 2006], which indicates the existence of other mechanisms for translational repression.

2.3.3.5. miRNA can cause mRNA association with P-bodies components, involved in translational repression.

P-bodies (processing bodies) are the aggregates of translationally repressed mRNAs, containing proteins involved in the inhibition of translation and mRNA degradation (Fig. 6F). The amount of translationally repressed mRNA positively correlates with an increase in size and in number of P-bodies, and vice versa [Teixeira D et al., 2005]. P-bodies contain a number of conservative "core" proteins, which include RNA decapping enzymes Dcpl/2, decapping activators Dhhl / RCK / p54, Pat1, Scd6 / RAP55, Edc3, Lsml-7 and 5'-3' exonuclease Xrnl [Parker R et al., 2007], as well as additional proteins found only in some organisms (e. g. GW182 protein in Drosophila and TNRC6 in humans). The P-bodies lack the translational initiation factors (with a few exceptions), and 60S ribosomal subunits [Valencia-Sanchez MA et al., 2006]. It is believed that a significant part of translational repression in the cell can be explained by P-bodies function [Parker R et al., 2007]. The mechanism of translational repression in P-bodies is still not well understood. However, it is clear that the association of target mRNAs and P-bodies components is required for repression [Bhattacharyya SN et al., 2006; Liu J et al., 2005b]. Taking in mind the absence of entire ribosome in P-bodies, it is believed that this repression occurs at the stage of translational initiation. mRNA can exit from P-bodies, enter the polysomes and be translated, and various

protein factors may shift this dynamic equilibrium in one or another direction [Bhattacharyya SN et al., 2006].

In most cases, the knockdown of P-bodies protein components disrupt the miRNA-dependent repression [Behm-Ansmant I et al., 2006; Chu CY et al., 2006; Jakymiw A et al., 2005; Liu J et al., 2005a]. In the composition of P-bodies Ago and miRNAs are found, which indicates the link between the P-bodies and miRNA-dependent silencing [Behm-Ansmant I et al., 2006; Bhattacharyya SN et al., 2006; Humphreys DT et al., 2005; Jakymiw A et al. 2005; Leung KM et al., 2006; Liu J et al., 2005b; Meister G et al., 2005]. Ago proteins interact with many components of P-bodies [Liu J et al., 2005a; Liu J et al., 2005b]. In Drosophila cell culture, the mutations that disturb the interaction between Ago1 and a key component of P-bodies (i.e. protein GW182), interfere with miRNA-dependent silencing [Eulalio A et al., 2009a]. Thus, Ago proteins can cause the translational repression, holding the mRNA in P-bodies and thereby associating mRNA with P-bodies components.

MiRNA targets form the basis of P-bodies, because the general disturbance of miRNA-dependent silencing in the cell leads to the disappearance of P-bodies [Pauley KM et al., 2006]. mRNA association with P-bodies components is accompanied not only with suppressing of its translation, but in some cases, by degradation.

However, the role of the macroscopic P-bodies in the repression of translation remains controversial. Several studies have shown that a mRNA association with P-bodies is not sufficient for the repression. A mutated GW182 that continues to interact with Ago1, but is not localized to macroscopic P-bodies, leads to the disappearance of macroscopic P-bodies, but does not disturb miRNA-dependent silencing [Eulalio A et al., 2009a]. The knockdown of some P-bodies proteins, resulting in the visual dissociation of P-bodies, has no effect on miRNA-dependent silencing [Chu CY et al., 2006; Eulalio A et al., 2007a]. This indicates that for the suppression of translation, the entering of mRNA target into macroscopic P-bodies is not necessary, and only association with P-bodies proteins is important.

The suppression of translation with the help of P-bodies can be the bridge that will unite, at least partially, the conflicting data on the mechanisms of miRNA-dependent translational repression. Firstly, the mechanism of repression via P-bodies is currently unknown. Secondly, a deadenylation of target mRNA observed in some cases as a response to the miRNA, may be associated with P-bodies. In Drosophila, the protein Ago1 binds to GW182 and inhibits the translation of mRNA through deadenylation [Iwasaki S et al., 2009]. On the other hand, the

protein Ago2 that does not interact with GW182, suppresses the translation by inhibiting the formation of initiation complex eIF4F, which indicates a non-exclusive role of P-bodies in the repression process [Iwasaki S et al., 2009].

2.3.3.6. Translational repression at the post-initiation step.

As it was mentioned above, two studies using IRES have indicated that that only cap-dependent, but not IRES-dependent translation is sensitive to miRNA-dependent repression, probably via the initiation factor eIF4E, and that miRISC may disrupt the binding between the initiation complex and the capped mRNA [Humphreys DT et al., 2005; Pillai RS et al., 2005]. However, another work using the same IRES had shown that the translation controlled by the same IRES was sensitive to RISC-dependent inhibition [Petersen CP et al., 2006]. As these IRES do not require any canonical translation initiation factors, these authors concluded that RISC suppresses the translation at some stage after the initiation.

How is it possible to explain the contradictory results obtained using IRES? In the first two studies, a transfection of in vitro synthesized mRNA was used as a model [Humphreys DT et al., 2005; Pillai RS et al., 2005]. In the third work, the cell culture was transfected with a plasmid encoding the mRNA of interest [Petersen CP et al. , 2006]. It is assumed that the mechanism of translational repression may depend on proteins associated with the target mRNA. The contradictions in the results mentioned above can be explained by the fact that in vitro synthesized mRNA, in contrast to the mRNA of nuclear origin, does not carry the associated protein factors, defining the mechanism of repression at post-initiation steps. This hypothesis suggests that the mechanism of repression of mRNA translation can depend on the promoter, which is responsible for the transcription of this mRNA [Kong YW et al., 2008]. Apparently, the promoter may determine the binding of different nuclear protein factors to mRNA, which determine the mechanism of repression. These contradictions highlight the importance of appropriate experimental models while studying miRNA-dependent repression.

Other studies revealed that repressed mRNA can stay in the polysomal fractions. In HeLa cells, a reporter mRNA, both repressed and non-repressed by a miRNA let-7a, is found in sucrose gradient polysomal fractions [Nottrott S et al., 2006], as well as, Ago2 and miRNA let-7a. Moreover, it was shown that in growing HeLa cells, the majority of miRNA colocalizes with polysomes [Maroney PA et al., 2006]. Thus, it was assumed that the mechanisms of miRNA-induced repression may include the degradation of newly formed protein or the reducing of the translation elongation speed (Fig. 6 A, B).

2.3.3.7. Slicer-independent degradation of mRNA.

In the first studies of miRNA, it was assumed that the main method of repression in animals is the translation repression without a noticeable decrease in the level of mRNA target. However, in recent years, a considerable number of observation of miRNA-dependent degradation of mRNA was published [Behm-Ansmant I et al., 2006; Chendrimada TP et al., 2007; Eulalio A et al., 2007b; Giraldez AJ et al., 2006; Wu L et al., 2005; Wu L et al., 2006]. Much of this degradation occurs in the absence of complementarity in the middle of mRNA and miRNA duplex. The using of microarrays for full transcriptome search of target sites which cause miRNA-dependent degradation of mRNA in human cell culture, shows that the vast majority of these sites forms a complementary pair with only seed region, but not with 10-11 nucleotides within miRNA [Grimson A et al., 2007]. This indicates that a slicer-independent mRNA degradation is widespread. The existence of such degradation is confirmed by experiments, in which the degradation of reporter mRNA carrying boxB sites, could be caused by miRNA-independent binding of the transgenic protein Ago1- λ N [Eulalio A et al., 2009b].

This raises a question about the mechanism of slicer-independent mRNA degradation. For degradation of mRNA in eukaryotic cells, there are two conservative ways; both of them promote the removal of the mRNA poly(A) tail as the first step [Parker JS et al., 2004]. The mRNA is then degraded by the exosomes in the 3'-5' direction, or undergoes decapping and 5'-3' degradation in P-bodies. The components of P-bodies that are attracted to the mRNA by miRlSC inhibit the translation initiation of mRNA. In the embryos of D.rerio, of C.elegans, as well as in Drosophila and human cell culture, miRNA enhances the deadenylation and decapping of their targets [Behm-Ansmant I et al., 2006; Eulalio A et al., 2007b; Giraldez AJ et al., 2006; Wu L et al, 2006].

In the culture of Drosophila cells, the changes in the level of 6000 mRNA were studied using oligonucleotide microarrays, in response to the protein knockdown of Ago1, Drosha, deadenylating, decapping enzymes and protein GW182. It was shown that the correlation between the sets of mRNA, that increase after the knockdown of Ago1 and GW182, as well as of deadenylating enzymes CAF1 and NOT1, can be esteemed as 60-75% [Behm-Ansmant I et al., 2006; Eulalio A et al., 2009b]. Such level of correlation is observed after the knockdown of Ago1 and Drosha, belonging to the same regulatory pathway [Rehwinkel J et al., 2006]. Consequently, the deadenylating enzymes CAF1 and NOT1, as well as GW182 protein, are involved in almost all cases of miRNA-dependent mRNA degradation in Drosophila cell culture. After the knockdown of Ago1 and of decapping enzymes Ge-1, Dcp1

and HPat, the correlation is only 15% [Eulalio A et al., 2007b], but the role of decapping may be higher, because the protein Dcp2, which is the main decapping enzyme in Drosophila, was not included in this study. The protein GW182, which is essential for slicer-independent degradation of mRNA, is a component of P-bodies [Parker R et al., 2007]. miRISC attracts the components of P-bodies (including GW182) to the mRNA and, taking into account that the decapping enzymes and 5'-3' exonuclease are present between these components, it is logical to assume that the slicer-independent degradation of mRNA occurs due to the association of mRNA with the components of P-bodies.

In the future, it is necessary to answer a number of questions. What governs the choice between the suppression of translation and a slicer-independent degradation of mRNA? Is a slicer-independent degradation of the mRNA a side consequence of the association of translationally repressed mRNAs with the components of P-bodies, or it is an independent mechanism? Why some mRNAs are subject to such degradation, and some are not? Probably, the attempts to answer these questions will reveal new roles for regulatory proteins that bind to specific sequences in mRNAs and defining the repression mechanism.

2.3.3.8. miRNA-dependent activation of translation.

The arrest of cell cycle in mammalian cell culture leads to the activation of translation of mRNA TNF-a, as well as of some reporter constructs, due to the miRISC complex binding, which contains the Ago2 and FXR1 proteins [Vasudevan S et al., 2007; Vasudevan S et al., 2007]. The activation of translation after a cell cycle arrest is induced by miRNA let-7 and by CXCR4, which normally induce translational repression in the proliferating cells. It is assumed that a formation of two types of miRISC, repressive or activating, is possible depending on the intracellular conditions. However, the data on miRNA-induced translational activation was obtained only by one group of researchers, and more studies are needed to evaluate the frequency and the importance of this phenomenon.

2.3.4. A comparison of miRNA and siRNA-dependent silencing.

The gene multiplicity of Ago as well as Dicer in some cases, can allow to divide the ways of miRNA and siRNA-dependent repression for their differential regulation. In plants, there are 4 genes encoding Dicer, and 10 genes encoding proteins of the Ago family; in Drosophila - 2 and 5, in mammals - 1 and 7 (8) of genes, respectively [Siomi H et al., 2009]. In Drosophila, Dicer2 and Ago2 participate mainly in the siRNA pathway, whereas Dicer1 and Ago1 - in the miRNA pathway. Dicer2 cuts the dsRNA and Dicer2/R2D2 complex loads the newly formed

siRNA in Ago2 [Forstemann K et al., 2007; Tomari Y et al., 2007]. Drosha/Pasha and Dicer1/LOQS complexes are involved in the formation of miRNA, and the unknown complex loads miRNA in Ago1 [Denli AM et al., 2004; Forstemann K et al., 2007; Forstemann K et al., 2005; Tomari Y et al., 2007]. In the study of RNAi in « in vitro » system, there were described the properties of effector complexes containing Ago2 and Ago1, and it was shown that both siRISC and miRISC can cause the degradation of the complementary target mRNA [Okamura K et al., 2004]; however, miRISC performs this with much lower efficiency [Forstemann K et al., 2007; Tomari Y et al., 2007].

In mammals, the division of siRNA and miRNA pathways is not observed. It is shown that human Ago1, 2 and 3 in vivo can bind both siRNA and miRNA [Liu J et al., 2004]. The experiments on embryonic stem cells show that all mammalian Ago are capable for the miRNA-dependent inhibition of translation [Su H et al., 2009]. This may be explained by the presence of a single protein Dicer, which participates in biogenesis as well as in loading of the both types of short RNAs into RISC. The redundancy of Ago genes in mammals may serve to suppress the expression with various RISC through various mechanisms, i.e. the degradation of target mRNA, the suppression of transcription, or the translation. Indeed, only the human RISC, carrying Ago2, is capable to promote the degradation of target mRNA [Liu J et al., 2004; Meister G et al., 2004]. Apparently, Ago1 and Ago4 are not able to cut the target due to a loss of His residue for Ago1 as well as one of Asp for Ago4 in the catalytic triad. The lack of Ago2 does not prevent the translational repression of a reporter gene in cell culture [Liu J et al., 2004]. This indicates that the proteins Ago1, Ago3 and/or Ago4 are involved only in the suppression of translation, but not in mRNA degradation. At the same time, the Ago1 protein is essential for the transcription silencing induced by siRNA, which is homologous to the promoter of the reporter gene [Kim DH et al., 2006].

3. The role of the 3 'UTRs of mRNA in the regulation of translation.

The attention of researchers to the 3 'untranslated region of the mRNA has appeared relatively recently [Standart N et al., 1994, as one of the first review on this topic]. The role of 3 'UTR in a post-transcriptional regulation of gene expression is due to the effect on mRNA stability, its localization in the cell and/or translation efficiency. This part of the Introduction will focus on the participation of 3' UTRs in the regulation of translation.

3.1. Main steps in eukaryotic translational initiation.

Usually, the rate-limiting translation step is initiation and as a rule, it is mostly susceptible to the regulation involving the 3' UTRs of mRNA. Some mRNAs can initiate translation in a cap-independent manner, where ribosome is recruited to the correct initiation codon by structural motifs in the 5'-untranslated region - internal ribosome entry sites (IRES), that is recognized by activated 40S initiation complex, followed by a mRNA scanning to the initiator codon. Cap-independent initiation requires additional proteins such as La-protein (a polypyrimidine-binding protein, PTB). However, the majority of eucaryotic mRNAs initiate translation by a mechanism that depends on the recognition of the m7G(5′)ppp(5′)N structure (cap) located at the 5′ end of most mRNAs. The complicated process of translation initiation in eukaryotes consists of several stages (Fig. 7) involving a large number of eukaryotic initiation factors (eIFs). The 5′cap structure is recognized by eIF4E alone (in this case, this event is followed by a heterotrimeric eIF4F complex formation i.e. association of eIF4E with scaffold protein eIF4G and the RNA helicase eIF4A) or in a composition of eIF4F preformed complex. Additionally, eIF4G interacts with eIF3 and with the poly(A)-binding protein (PABP) that is bound to the poly(A) tail of the mRNA, leading to mRNA circularisation. Translation initiation factor eIF4A promotes the ATP - dependent melting of secondary structures in the 5 'UTRs of mRNA with the help of elF4B, the eIF4A ATPase center activator. In the next step, the 5 'end of the mRNA joins 43S preformed preinitiation complex, consisting of a small 40S ribosomal subunit as well as the initiator Met-tRNAi in complex with eIF2 - GTP and several other translation initiation factors indicated in Fig.7. This mRNA binding is achieved probably via eIF3, that can interact with both eIF4G and 40S ribosomal subunit, and is stabilized by eIF1A. At the next step, 43S complex scans the 5 'UTRs to the initiation codon in an appropriate sequence context (the Kozak context), leading to the formation of the 48S initiation complex. At this step, eIF1 is displaced and eIF5 mediates the hydrolysis of eIF2-bound GTP. At the final stage of translation initiation, there is an accession to the 48S preinitiation complex of a large 60S ribosomal subunit resulting in 80S complex formation. This process is promoted by eIF5B and is accompanied by the hydrolysis of another GTP molecule. All above-mentioned steps can be regulated during translation initiation through the 3' UTRs of mRNA. Naturally, the question arises what influence has a 3 'untranslated region, as well as its associated proteins, upon the events taking place at the 5' end of the mRNA molecule. The regulation models proposed in literature are based on the mRNA circularization.

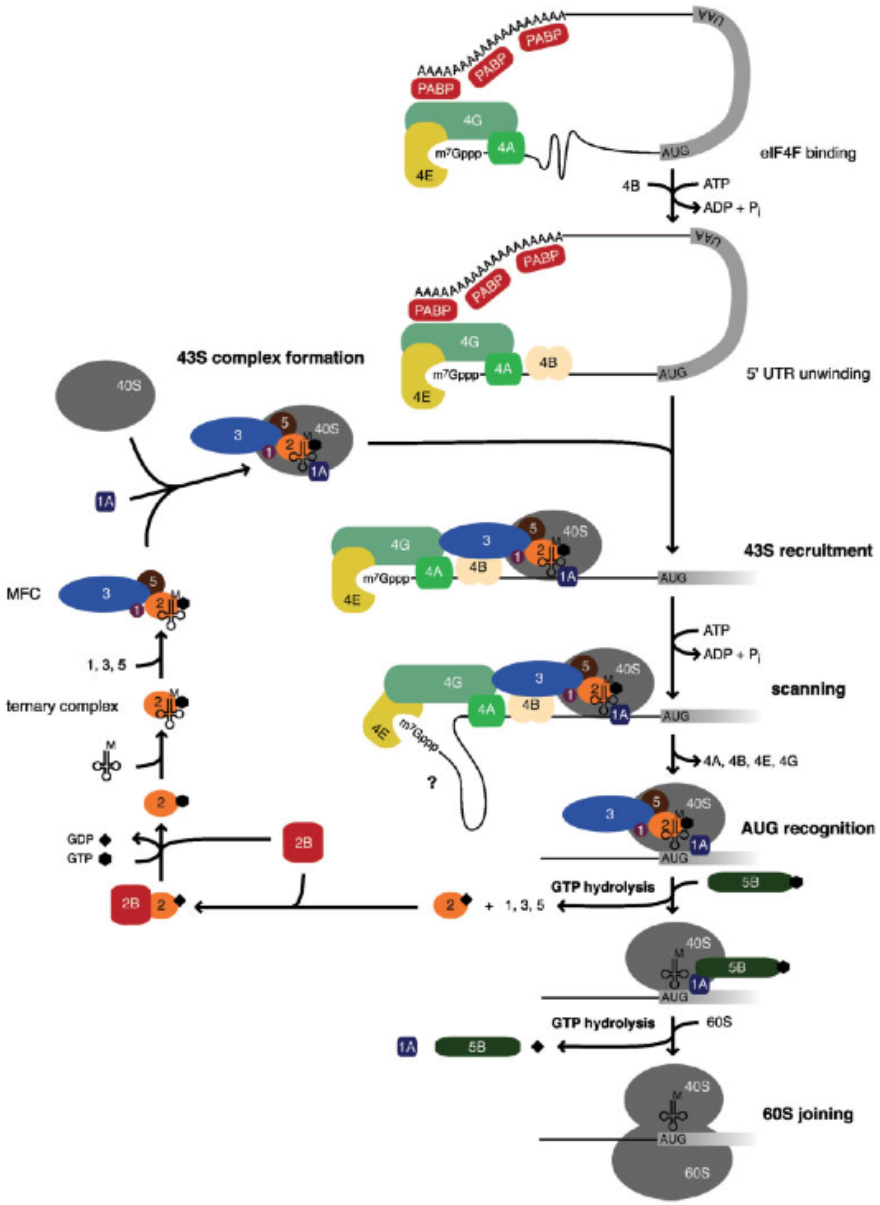


Figure 7. Main steps in eukaryotic translational initiation [from Preiss and Hentze, 2003, with changes].

3.2. Regulation involving proteins that interact with the 3`UTR of mRNAs.

The hypotheses about mRNA circularization during translation have been proposed long time ago. The most popular of them is based on the fact that the poly(A) - binding protein associated with the 3'-terminal poly(A) tail of a mRNA interacts with initiation factor eIF4G, forming the part of a translation initiation complex eIF4F, which binds to the 5 'end of the mRNA molecule [Tarun SZ et al., 1996; Wells SE et al., 1998]. It is believed that the

formation of a closed mRNA ring promotes the recycling of ribosomes and, consequently, the protein synthesis efficiency. This model explains many experimental data [for example, Sachs, 2000], but it has a number of controversies, as well [Kozak M et al., 2004]. Thus, it was assumed that at the basis of the stimulating effect of poly(A) - binding protein on the translation under the hypotheses described above, there is the interaction of RNA-recognition PABP motifs RRM1 and RRM2 with the initiation factor eIF4G, and the destruction of this interaction abolishes the stimulating effect of PABP on the translation. However, some experiments show that PABP with deleted RRM1 and RRM2 is unable to interact with eIF4G, but can nevertheless stimulate the reporter mRNA translation. In addition, PABP is able to activate the translation of mRNAs without a poly(A) tail, and therefore, PABP may operate without contacting the poly(A) tail [Otero LJ et al., 1999].

Although these and some other facts put into question the current version of the mRNA circularization hypothesis, this model was very attractive for the researchers who have tried to explain the effect of 3'UTRs on translation of a particular mRNA, by two reasons. Firstly, when mRNA circularization appears, 3' UTRs becomes close to the 5' end of the mRNA, and proteins that interact with the 3'UTR could directly affect the initiation of translation. Secondly, in addition to the above-described circularization, mRNAs may form an alternate circular structure involving proteins associated with the 3 'UTRs and 5' UTRs of mRNA molecule. In this case, it is possible to obtain either activation or inhibition of translation.

The most striking examples of such translational control are obtained in studies of early embryonic development. Thus, it is shown that during the maturation of Xenopus leavis oocytes, the synthesis of key proteins of this process, c-Mos and cyclin B1, is determined by polyadenylation of their mRNAs (Fig. 8) [Sheets MD et al., 1994]. The mRNAs of these proteins have a short poly(A) tail and are found in masked state in immature oocytes. There are two regulatory sequences in their 3 'UTRs, such as CPE (cytoplasmic polyadenylation element) which specifically interacts with the protein CPEB (CPE-binding protein), as well as a hexanucleotide polyadenylation signal (AAUAAA), recognizable by CPSF (cleavage and polyadenylation specificity factor). A complex formed on the 3' UTRs includes the other proteins as well. A scaffold protein Symplekin interacts with both CPEB and CPSF. The Poly (A) polymerase Gld-2 is included in the complex through an interaction with CPSF [Barnard DC et al., 2004]. CPEB in its non-phosphorylated state binds PARN deadenylase and protein Maskin. The colocalization of poly(A) polymerase and PARN deadenylase leads to the maintenance of short poly(A) tail of target mRNAs. This fact contributes to the translation inhibition [Kim JH et al., 2006; Kuersten S et al., 2003]. However, it is believed that the main

role in the inhibition is played by Maskin. This protein has a high affinity for the cap-binding protein eIF4E. It replaces eIF4G in the complex containing eIF4E, which leads to the inhibition of translation and mRNA circularisation through Maskin (5`cap-eIF4E-Maskin-CPEB-CPE-3 'UTRs of mRNA) [Stebbins-Boaz B et al., 1999].

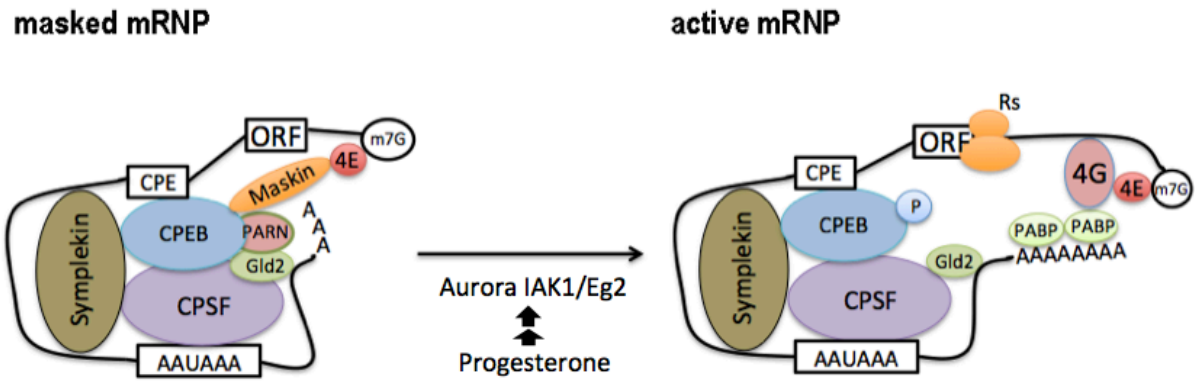


Figure 8. Model for translational inhibition and activation of c-mos and cyclin B1 during maturation of oocytes in X. leavis [from Kim JH et al., 2006, with changes].

During maturation of oocytes after progesterone stimulation, CPEB is phosphorylated by the kinase Aurora IAK1/Eg2 on Ser-174 or Thr-171, which leads to the increasing of affinity of CPEB to CPSF, as well as to a recruitment of the poly(A) polymerase GLD-2 and to PARN deadenylase dissociation [Tay J et al., 2003 ; Villalba A et al., 2011 ; Fernandez-Miranda G et al., 2012]. In such complex, the synthesis of poly(A) tail occurs without a subsequent shortening by deadenylase. The newly synthesized poly(A) sequence binds PABP, which in its turn interacts with eIF4G. This event increases the affinity of eIF4G to eIF4E, which leads to the displacement of Maskin complex from eIF4E. The displacement of Maskin is probably influenced by the destabilization of its interaction with CPEB after the CPEB phosphorylation [Sarkissian M et al., 2004]. As a result of all these events, the classic circular structure occurs (with PABP, eIF4G, eIF4E), which induces an efficient translation of mRNA [de Moor CH et al., 1999; Groisman I et al., 2002; Kim JH, 2006]. Similar regulatory system has been found in the translation studies of nerve cells axons. It is considered to play a key role in synaptic plasticity [Richter JD, 2001; Wu L et al., 1998]. The Maskin protein function in this situation is performed by another eIF4E-binding protein, Neuroguidin [Jung MY et al., 2006; Huang YS et al., 2007].

It is necessary to note that the translational inhibition model involving Maskin protein is not controversy-free. In addition to lack of certain direct experimental evidences [discussed in Kozak M, 2004; Kozak M, 2006], the main problem of the above-described scheme is that all Maskin homologs, even in species close to Xenopus laevis, lack the putative specific binding site to eIF4E. In addition, the role of Maskin in early embryonic development may be associated not only with the translation, but also with the microtubules organization in the cell [O'Brien LL et al., 2005]. In addition, recent studies show that in the case of CPEB-mediated inhibition of cyclin B1, Xp54 and Pumilio proteins can be key to the regulation of the cyclin mRNA translation. Xp54 is a RNA helicase, which interacts with CPEB and eIF4E, both directly and indirectly. These interactions, as well as the helicase activity of Xp54, are essential for translation inhibition [Minshall N et al., 2001]. It is interesting that after the polyadenylation of the cyclin B1 mRNA, the protein Xp54 is not included in the complex with 3 'UTRs of this mRNA, and the translation is not inhibited. How the protein Pumilio influences translation is even less clear, but it is believed that it contributes to the inhibition of translation initiation through the poly(A) tail shortening, recruiting a deadenylase complex to mRNA [Rouhana L et al., 2007]. However, these models are not yet sufficiently confirmed experimentally to describe them in more detail. At the same time, their appearance shows that in the near future, our understanding of the molecular mechanisms of translational regulation during the maturation of oocytes can become even more complicated.

The Drosophila eIF4E-binding protein Cup is involved in the translational repression of mRNAs oskar and nanos (Fig. 9). The protein Bruno provides its interaction with the 3 'end of the mRNA molecule in the case of oskar, [Kim-Ha J et al., 1995; Wilhelm JE et al., 2003] and protein Smaug does the same thing in the case of nanos mRNA [Dahanukar A et al., 1999; Nelson MR et al., 2004]. It is believed that in this way, the formed circular structures can interfere the interaction between eIF4E and other components of eIF4F initiation complex, and this provides a translational repression of oskar and nanos mRNAs (Fig. 9).

It was assumed that a Drosophila protein Bicoid, which contains the eIF4E binding motif (same as the previously-mentioned Maskin protein), prevented an interaction between eIF4E and eIF4G, and thereby inhibited the translational initiation of caudal mRNA (Fig.9) [Niessing D et al., 2002]. In this case, the mRNA was locked in a ring structure: 5'cap-eIF4E-Bicoid-BBR-3`. However, [Barnard DC et al., 2005] showed that the inhibitory complex included a protein having some structural similarity to eIF4E and named 4EHP (eIF4E-homologous protein). Due to some differences from eIF4E, this protein has a higher affinity

to Bicoid, than to the initiation factor eIF4G, but can still effectively interact with mRNA cap structure.

Surprisingly, mammalian 4EHP can also activate translation, for example, during hypoxia, illustrating the complexities of translational control [Uniacke J et al., 2012]. Being similar to 4EHP, CPEB also does not always function as a repressor (described above). So, both 4EHP and CPEB are the examples of RNA-binding proteins that can function either as repressors, or as activations of translation, depending on the cellular context, mRNA targets and protein partners.

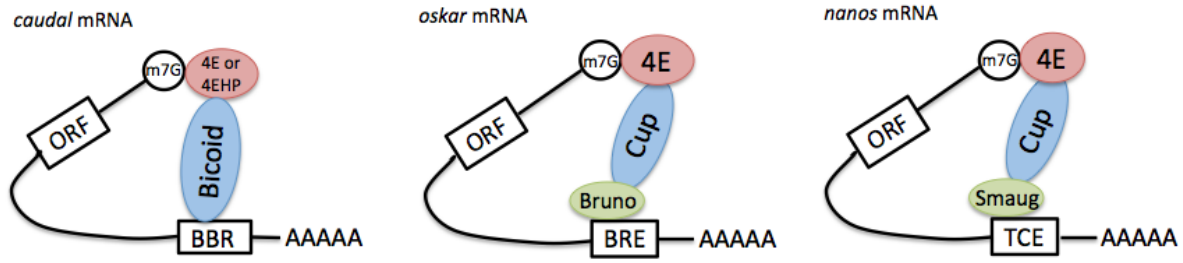


Figure 9. Maskin-related inhibitory complexes, regulating a translation of a key proteins of Drosophila embryogenesis.

However, translation initiation can be repressed without disrupting a classical closed-loop structure. The experiments performed on U937 cells have shown that 2-4 hours after stimulation of cells by interferon, the ceruloplasmin mRNA synthesis, and respectively the synthesis of the ceruloplasmin (Cp) itself, begins (Fig. 10A) [Mazumder B et al., 1997]. However, 24 hours later there comes the repression of mRNA translation of Cp [Mazumder B et al., 1999]. It was found that 3 'UTRs of Cp mRNA contains a 29-nucleotide regulatory site - GAIT-element (interferon-activated inhibitor of translation), which is associated with several proteins i.e. glyceraldehyde-3-phosphate dehydrogenase, phosphorylated glutamyl-prolyl tRNA synthetase (EPRS) and protein NSAP1 (hnRNP Q) [Mazumder B et al., 2005; Mazumder B et al., 2003]. This so-called pre-GAIT-complex does not interfere with normal flow of the translation initiation. 16-24 hours after cells stimulation with interferon, the pre-GAIT-complex interacts with the ribosomal protein L13a, which subsequently plays a role of translational repressor [Mazumder B et al., 2003]

This event is possible due to the fact that L13a is phosphorylated and leaves the 60S ribosomal subunit at this time (Fig. 10B). A necessary condition for repressor protein action is the interaction of three main elements that cause circularization of mRNA: poly(A) tail,

PABP and eIF4G [Mazumder B et al., 2001]. Due to the circularization of the mRNA, the L13a, bound to GAIT-complex, is in the close proximity to the translation initiation factor eIF4G. It is assumed that the formation of this contact prevents the eIF4G interaction with other initiation factor eIF3 (more precisely, with its eIF3e subunit). As a result, the 43S preinitiation complex binding to the mRNA is inhibited, leading to the transcript-specific translational repression without suppressing of the eIF4F complex formation (Fig. 10C) [Kapasi P et al., 2007].

In male Drosophila, the transcription complex MSL (Male Specific Lethal) participates in genes transcription enhancing on the single X chromosome (dosage compensation). The Msl genes of Drosophila females are also transcribed, but the expression of one of them, msl-2, is suppressed at post-transcriptional level by the repressor protein SXL (sex-lethal) (Fig. 11). The Protein SXL, specific to females, binds within the 5 'UTR of msl-2 mRNA with the two regulatory U-rich regions. Furthermore, SXL in a complex with the protein UNR interacts with four regulatory regions in the 3`UTRs of msl-2 mRNA. The combined effect of these protein complexes, bound to the 3' and 5' untranslated regions of the same mRNA, prevents the recruitment of 43S preinitiation complex, as well as 5' UTR scanning [Abaza I et al., 2006; Gebauer F et al., 1999; Gebauer F et al., 2003].

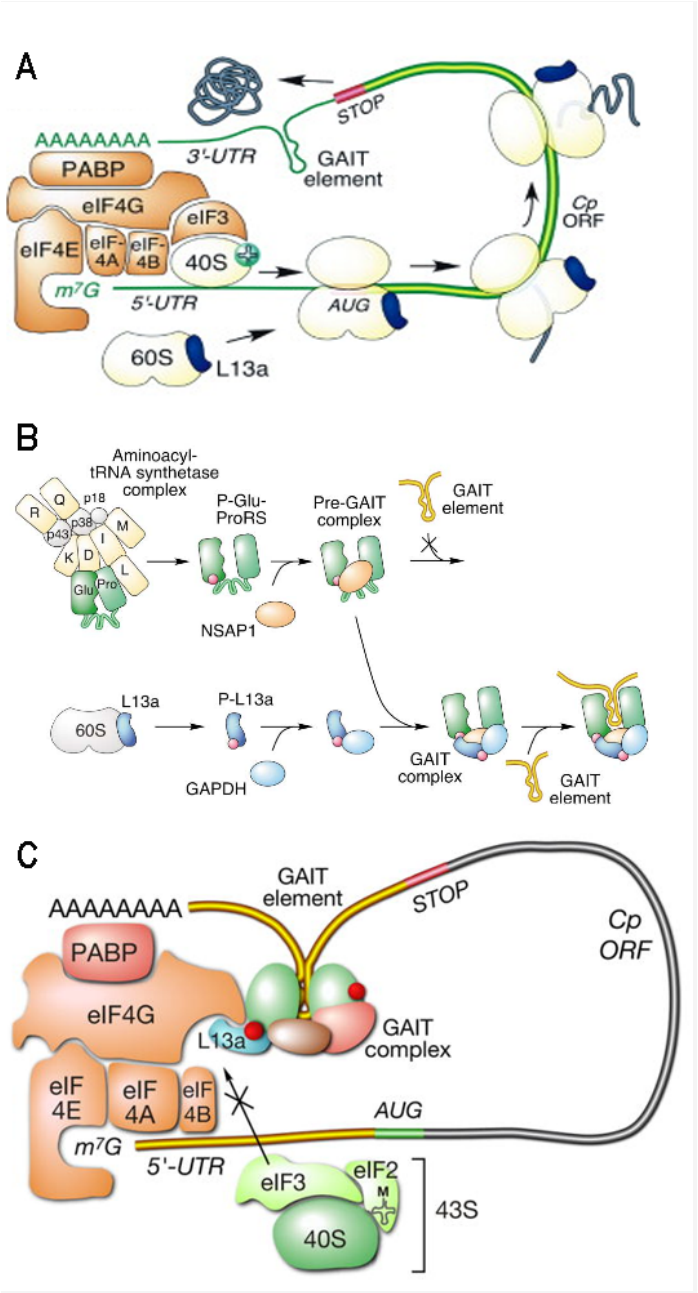


Figure 10. Transcriptional regulation of ceruloplasmin mRNA.

A. 4-8 hours after cell treatment with IFN-gamma. Ceruloplasmin mRNA is effectively translated.
B. 8-24 hours after cell treatment with IFN-gamma. L13a phosphorylation and GAIT-complex formation.
C. ~24 hours after cell treatment with IFN-gamma. GAIT-complex binds to ceruloplasmin mRNA. Due to the preserved mRNA circularization GAIT-complex is in close proximity to the translational initiation region.
L13a protein in the composition of GAIT-complex inhibits the translation of ceruloplasmin mRNA by preventing eIF4G and eIF3 interaction [from Kapasi P et al., 2007; Mazumder B et al., 2003; Sampath P et al., 2004, with changes].

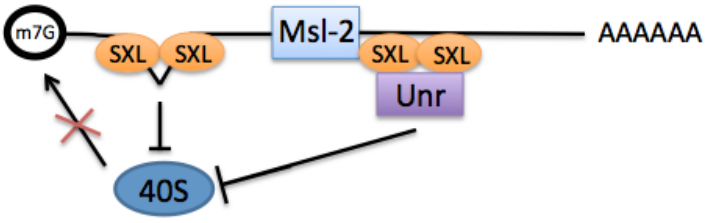


Figure 11. Translational inhibition of msl-2 mRNA [Abaza I et al., 2006; Gebauer F et al., 1999; Gebauer F et al., 2003].

An increasing number of RBPs is associated with multifunctional deadenylase complexes, leading to poly(A) tail shortening and mRNA destabilization, and/or to disruption of the closed-loop structure and decrease of translational efficiency. Thus, a protein complex (Nanos, Pumilio, Brain tumor) binds to the NRE-element (nanos response element) within the 3 'UTR of Hunchback mRNA, inhibiting its translation (Fig. 12). The Brain tumor and Pumilio are homogeneously distributed throughout the whole embryo, unlike Nanos, which is concentrated in the posterior pole of the embryo [Kuersten S. et al., 2003; Murata Y et al., 1995]. To inhibit the Hunchback synthesis, all three essential proteins are needed, that is why an effective inhibition of hunchback mRNA translation occurs only in the posterior pole of the embryo. The inhibition of hunchback mRNA translation occurs, apparently, through two mechanisms such as mRNA deadenylation [Wreden C et al., 1997] together with a mechanism similar to the inhibition of caudal mRNA (Fig. 12). Pumilio recruits the POP2/CCR4/NOT deadenylase complex to promote mRNA repression. It has been shown that the Pum/Nos/Brat complex, bound on 3' UTR, also recruits 4EHP to inhibit translation at the 5'-end of hunchback mRNA [Barnard DC et al., 2005]. It is therefore assumed that in this case, a removal of eIF4F initiation complex from the mRNA cap structure also takes place.

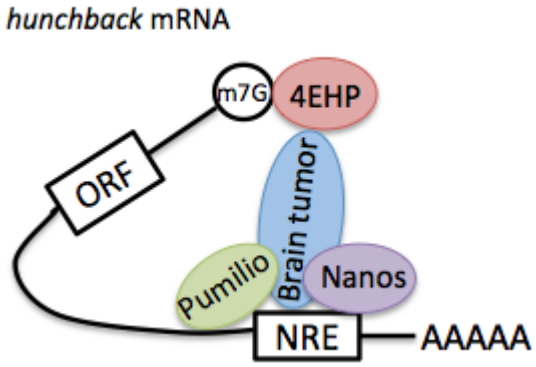


Figure 12. Translational inhibition of hunchback mRNA [Barnard DC et al., 2005].

All these examples offer interesting models of the 3' untranslated regions impact on translation initiation. There are many examples where the regulation takes place after closed-loop formation, targeting the step of 60S subunit binding. Thus, ZBP1 (zip-code-binding protein 1), which binds to the 3′UTR of β-actin mRNA and localizes it to the leading edge of fibroblasts or neurite growth cones, inhibits the translation of the β-actin mRNA during its transport. The ZBP1 blocks the 60S joining to the 43S complex [Huttelmaier S et al., 2005]. Another example of the same mechanism was the lipoxygenase mRNA, where it was shown that binding of hnRNP K and hnRNP E1 to the differentiation control element (DICE) on its 3' UTRs leads to the establishment of translational repression in the early stages of reticulocytes maturation (Fig. 13) [Ostareck DH et al., 1997]. In this case, the attachment of

60S ribosomal subunit to the 48S preinitiation complex was inhibited [Ostareck DH et al., 2001]. Later it was found that during maturation of reticulocytes, the hnRNP K protein is phosphorylated by Tyr kinase c-Src and loses its affinity to the DICE regulatory element within the 3 'UTRs of lipoxygenase [Ostareck-Lederer DH et al., 2002]. As a result, the translational block is removed, and the newly synthesized lipoxygenase triggers one of the last stages of reticulocytes maturation, i.e. degradation of mitochondria. Despite the elegance of this model, significant disadvantages can be noted. In particular, there is no evidence of lipoxygenase mRNA circularization, which could have explained the impact of 3 'UTRs on the translation initiation. Additionally, the inhibition of reporter DICE-containing mRNAs translation may occur even in the absence of poly(A) tail, as well as during a cap-independent translation initiation [Ostareck DH et al., 1997]. Thus, it is not clear how the contact between the inhibitory complex and mRNA 5 'end is achieved. Some critical-minded researchers may reasonably have a doubt about an accuracy of determining the stage of translational inhibition. For example, the experiments do not show any 48S intermediates accumulation, which should be observed during the inhibition of 60S ribosomal subunit joining [Ostareck DH et al., 2001].

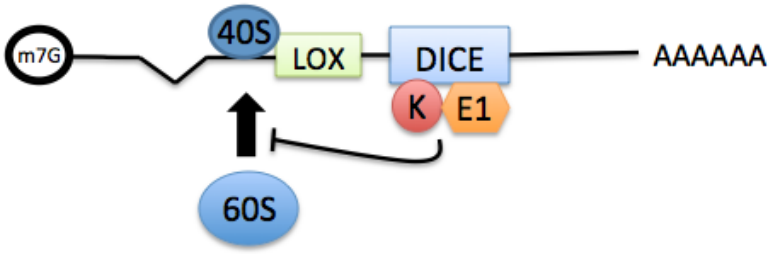


Figure 13. Translational inhibition of lipoxygenase mRNA [Ostareck DH et al., 1997].

Histone mRNAs are remarkable their absence of poly(A) tails. It is assumed that their function can be performed by the hairpin elements on 3 'UTRs as well as its specifically associated SLBP proteins (stem-loop binding proteins). These components are necessary mainly for processing and stabilizing of histones mRNAs [Dominski Z et al., 2007; Dominski Z et al., 2003]. It is believed that they also contribute to mRNA circularization due to the interaction between SLBP and translation initiation factors eIF4G and eIF3, located at the 5 'end of the mRNA molecule [Ling J et al., 2002]. This, in its turn, can contribute to more efficient translational initiation. However, at the moment there is no evidence of direct interaction between SLBP and elF4G, as well as eIF3, and besides, there are contradictions in the results obtained by different research groups. Thus, in one laboratory, SLBP stimulates

only non capped reporter mRNA with histone 3 'UTR [Sanchez R et al., 2002], and according to the experiments of another laboratory, it stimulates only the capped mRNA [Coles LS et al., 2002]. These data put into question the histone mRNA circularization hypothesis.

Although the most of RBPs have their influence on the initiation step of translation, some of regulators may target the elongation step. PUF family proteins (Pumilio and FBF) are reported to be associated with Argonaute (AGO) and the translation elongation factor eEF1A [Friend K, et al. 2012]. The PUF/AGO complex, bound to the 3'UTR, inhibits the eEF1A GTPase activity, leading to an inhibition of translation elongation. HnRNP E1 can interfere with different steps of translation depending on the associated factors. For example, being bound to eEF1A, it attenuates the elongation by blocking eEF1A dissociation from the ribosome [Hussey GS et al., 2011].

3.3. Interplay between RNA-binding proteins and miRNAs on 3` UTR.

3' UTR of a mRNA, playing an important role in post-transcriptional regulation of gene expression, controls this expression via a cross-talk between structural components of mRNA (cis-elements] and trans-acting factors (miRNA or RBPs). These molecules can bind to non-overlapping binding sites, or to common sites, in a competitive manner, within a mRNA target. RBPs and miRNA can regulate mRNA translation and stability in positive or negative manner. At the same time, they can cooperate or be antagonists in their actions, and the final collective result depends on many circumstances, such as their expression levels, affinity to the binding sites, and localization in the cell, which can be controlled by various physiological conditions.

3.3.1. Cooperation between miRNA and RBPs on 3`UTR.

RBPs actively participate in miRNA-dependent regulation of gene expression. For example, Pumilio proteins (PUM1 and PUM2) collaborate with several miRNAs and act as guides that mediate the opening of mRNA secondary structure, allowing interactions between miRNAs and their binding sites. A comparative study of Pumilio target sites within mRNA, using RIP-ChIP and PAR-CLIP in human cells, showed an enrichment for miRNA recognition sites in the sequences surrounding Pumilio binding sites, suggesting a widespread interaction of human Pumilio proteins with miRNA regulatory systems [Galgano A. et al., 2008].

Kedde and collaborators [Kedde M et al., 2010] have demonstrated in the MCF7 breast cancer cell line, that Pumilio promotes the inhibitory effects of miR-221/222 on the p27Kip1 mRNA by opening the secondary structure of the p27Kip1 3'UTR, allowing the access of miR-

221/222 to its binding site.

An elegant model was proposed for the HuR and let-7 cooperative action on the c-Myc 3' UTR [Kim HH et al., 2009]. HuR bind the 3' UTR of c-Myc mRNA in a position near let-7 binding site and reduces c-Myc expression. The activity of let-7 is dependent on HuR, and the activity of HuR depends on let-7. The authors discovered that HuR binding changes the local mRNA conformation and promotes the association of let-7 with the c-Myc mRNA, by unmasking let-7 recognition site.

RBPs can directly interact with AGO and recruit RISC.

RBPs and RISC complex can have a direct physical interaction. In an early study, Caudy and colleagues demonstrated that the RISC complex in Drosophila contains proteins that associate with AU-rich elements, i.e. the protein FXR1 [Caudy AA et al., 2002]. Reduction of FXR1 in Drosophila S2 cells resulted in less efficient RNAi silencing. Further work confirmed that FXR1 associates with AGO2 in mammalian cells as well [Jin P et al., 2004]. The discovery of a functional interaction between FXR1 and Ago2 resulted in suggestions that either RBPs are targeted to substrates by the RNAi complex [Caudy AA et al., 2002], or that FXR1 scans for the G quartet, previously reported to be a recognition site for FXR1, and then recruits RISC.

3.3.2. RNA-binding proteins counteract miRNA function on 3`UTR.

The miRNA activity can also be inhibited by RBPs bound to target 3'UTRs via a 'competition for binding sites' mechanism. DND1, an RNA-binding protein that mediates germ-cell viability and suppresses the formation of germ-cell tumors [Kedde M et al., 2007] has been shown to interact with the 3' UTRs of p27Kip1 and LATS2 mRNAs. DND1 binds these mRNAs and interferes with the function of miRNAs, helping to maintain p27Kip1 and LATS2 (tumor suppressors) protein levels in a germ-cell tumor cell line and in squamous cell carcinoma, thus inhibiting tumorigenesis. Generally there are two mechanisms of DND1 action: DND1 binding may result in a mRNA secondary structure formation that makes miRNA recognition sites inaccessible, or alternatively, binding of DND1 could change a subcellular localization of the transcript, making it inaccessible to the RISC complex.

Another interesting example of antagonistic interactions between RBPs and miRNAs was demonstrated by Bhattacharyya SN and Filipowicz W. They performed an elegant study demonstrating that the miRNA-dependent repression is a reversible process. Downregulation of the cationic amino acid transporter 1 (CAT-1) mRNA by miR-122 was inhibited by stress, and stress-triggered elevation of cellular HuR, predominantly nuclear, allows its entrance in cytoplasmic P-bodies (transcript decay structures), its binding to the specific AU-rich

sequence on CAT-1 mRNA and to a relocalization of CAT-1 mRNA from P-bodies to polysomes [Bhattacharyya SN et al., 2006]. Recently, the same authors have demonstrated a possible mechanism of HuR action on CAT-1 3' UTR. Because HuR AREs are positioned at a considerable distance from the miRNA sites, HuR promotes the relief of miRNA repression from a distance by a process likely involving HuR oligomerization [Kundu P et al., 2012].
Therefore, RBPs can directly interact with RISC (RNA-induced silencing complex) complex, either stabilizing the binding of the latter to target mRNAs or antagonizing miRNA functions, depending on the mRNA-target and on the miRNA incorporated in RISC.

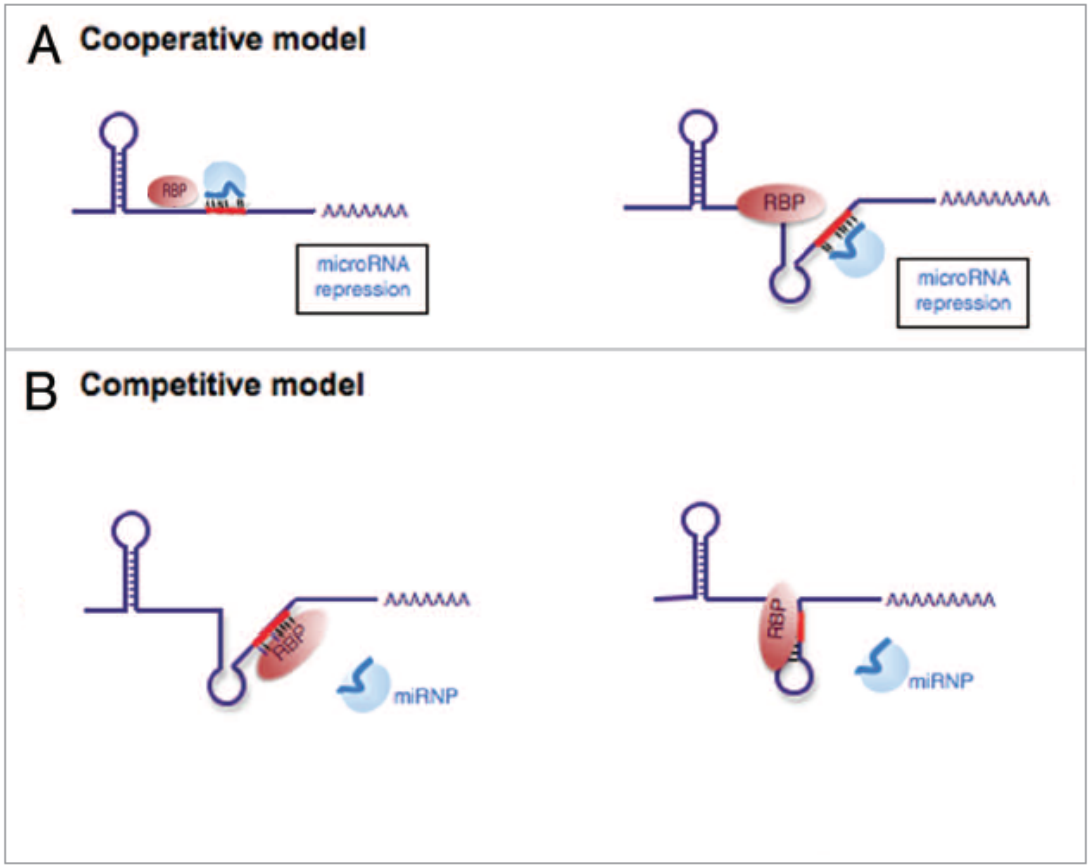


Figure 14. miRNA/RBP interplay in the regulation of the same mRNA [from Ciafrè SA et al., 2013, with changes].
A) Cooperation : on the left : RBP scan enhance miRNA function by opening a secondary structure, allowing the interaction between miRNA and it target site within 3`UTR.
On the right : RBP scan recruit RISC complex to the 3`UTR and stabilize its binding via direct interaction with RISC components.
B) Competition : RBP can interfere miRNA function via competition for the same binding site. In the context of non-overlapping sites RBP can change local mRNA structure and prevent miRNA binding.

RBPs, translational inhibitors or activators, can interact on mRNA in cooperative or antagonistic manner, regulating the rate of protein translation. Translational activators can bind the specific target sequences and/or secondary structures on 3`UTR of mRNA. Translational inhibitors can be recruited to mRNA 3`UTR via miRNAs, which bind to their specific sites. During our study of cyclin D1 and D3 regulation by IMP3, we observed a phenomenon of IMP3-RISC competition for cyclin D1 and D3 binding and translational regulation. We identified regions within cyclins 3`UTRs, targeted by this regulatory mechanism in vitro, and we proved their functional significance in vivo.

Previous observations show that human IMPs and their homologs from other organisms can « cage » their mRNAs targets, forming a cytoplasmic mRNPs with other RBPs, making these mRNAs inaccessible to RISC-dependent repression. Following this logic, we identified the exact composition of IMP3-containing mRNP complex, which can post-transcriptionally regulate cyclin D1 and D3 expression.

RESULTS

Article N° 1 (submitted). IMP-3 protects the mRNAs of cyclins D1 and D3 from GW182/AGO2-dependent translational repression.

Previous studies in my group have shown that all three IMP proteins can directly bind the mRNAs of cyclins D1, D3 in vitro, but only IMP-3 regulates the expression of these cyclins in a significant manner in vivo. In this study we tried to determine the molecular mechanisms of the regulation of the expression of cyclins D1 and D3 by the RNA-binding protein IMP3/IGF2BP3 in human cancer cells.

We have shown, that IMP-3 KD promotes decrease in cyclin D1 and D3 protein levels, whereas their mRNA levels do not change. In the absence of IMP3, Cyclin D1 and D3 mRNAs are exported to the cytoplasm and are observed within polyribosomal fractions, but not translated. Previous observation in my group have shown that IMP3 interact with a multiple RNA-binding proteins in human cancer cells. We found that some of them (HuR, PTBP1 and NF90) influence cyclin D1 and D3 protein levels and co-interact with cyclin D1 and D3 mRNAs, suggesting a formation of IMP3-containing complex on 3`UTRs of the cyclins. This complex can protect cyclin D1 and D3 from miRISC-dependent translational repression. Consistent with this hypothesis, we found that decrease of cyclin D1 and D3 protein levels after the inhibition of IMP3, NF90 or PTBP1 can be fully reversed after the simultaneous inhibition of two key components of miRISC : Ago2 and GW182/TNRC6. We concluded that IMP3 and RISC complexes can compete for cyclin D1 and D3 translational regulation. We proved that this regulation is direct by luciferase assays. Finally, we identified a functional regions within 3`UTRs of the cyclins, where a competition between IMP3 and miRISC complexes occurs.

In summary, our findings indicate that IMP3 and its protein partners NF90, PTBP1 form a complex on 3`UTRs of the cyclins D1 and D3, protecting them from miRISC-dependent translational repression.

IMP-3 protects the mRNAs of cyclins D1 and D3 from GW182/AGO2-dependent translational repression

Evgeny Deforzh[1], Thaiz Rivera Vargas[2], Anna Polesskaya[1,3#]

[1] Institute for Integrative Biology of the Cell (I2BC), IBITECS, CEA, CNRS, Université Paris‐Sud, Université Paris‐Saclay, 91198, Gif‐sur‐Yvette cedex, France

[2] INSERM; UMR 866; Centre Georges François Leclerc; Université de Bourgogne, Dijon, France

[3] Laboratoire de Biochimie, Ecole polytechnique, CNRS, Université Paris-Saclay, 91128 Palaiseau cedex, France

[#] corresponding author, e-mail: anna.polesskaya@cea.fr

Key words: IGF3BP3, IMP-3, RISC-mediated translational repression, cyclins

Running title: IMP-3 and RISC competition regulates cyclins expression

Abstract

IGF-2 mRNA binding protein 3 (IGF2BP3, IMP-3) is a well-known post-transcriptional regulatory factor of gene expression, mainly involved in embryonic development and oncogenesis. We have previously demonstrated that a subset of IMP-3 targets, such as the mRNAs of cyclins D1, D3, and G1, are positively regulated by IMP-3, and that this regulation depends on nuclear localization of IMP-3. In this study, we show that as a first step following a knock-down of IMP-3, the protein levels of the cyclins rapidly decrease, while their mRNAs remain stable and associated with the polyribosomes, though not translated. We have elucidated the molecular mechanisms of this regulation, demonstrating that IMP-3 and its protein partners ILF3/NF90 and PTBP1 bind to the 3'UTRs of the cyclins mRNAs and protect them from the translational repression induced by miRNA-dependent recruitment of AGO2/GW182 complex in human cancer cells.

Introduction

Post-transcriptional regulation of gene expression plays a very important role at all stages of normal development, maintenance and/or pathology of cells, tissues, and organs. Post-transcriptional regulation depends on various RNA-binding proteins (RBPs) that determine

the translational status of mRNAs, their structure, localizaton, and degradation rate. Regulatory RBPs can be recruited to their target mRNAs via specific recognition sequences (such as AU-rich sequences for HuR protein, or CPE sequence for CPEB family (Brennan and Steitz 2001; Richter 2007)). Alternatively, RBPs have been shown to bind to specifically structured elements within target RNAs, regardless of their nucleotide sequence. The IMPs/IGF2BPs (IGF-2 mRNA-binding proteins 1-3) are likely to belong to this latter group. IMP target sequences within RNAs vary to a large extent in different experimental models (Munro, Kwon et al. 2006; Jonson, Vikesaa et al. 2007; Chao, Patskovsky et al. 2010; Hafner, Landthaler et al. 2010). In addition, the ability of IMPs to form homo- and heterodimers further complicates the target sites identification and/or prediction, which is already extremely complex for the structure-recognizing RBPs (Nielsen, Nielsen et al. 2002). Finally, a large number of RNA-binding regulatory proteins are recruited to their target mRNAs via short non-coding RNAs, such as miRNAs (reviewed in (Filipowicz et al., 2008)). Cooperation or competition between different RNA-binding protein complexes determines the rate of protein expression from a large number of mRNAs (Ciafre and Galardi 2013)

Post-transcriptional regulation of gene expression is particularly important in the case of proteins characterized by a short half-life, which are often critical for the control of cell cycle, signal transduction pathways, circadian rhythm, antigen processing and other processes (Glickman and Ciechanover 2002). We have recently shown that IMP-3/IGF2BP3 protein binds to and positively regulates the expression of cyclins D1, D3 and G1 in a number of human cancer cell lines (Rivera Vargas, Boudoukha et al. 2014). This regulatory mechanism controls the cell cycle and proliferation. In the current study, we explore the molecular mechanisms underlying the regulation of the cyclins' expression by IMP-3 in human rhabdomyosarcoma cells.

Results and Discussion

The expression of CCND1 and D3 is regulated on post-transcriptional level in IMP-3 KD cells.

To elucidate the molecular mechanisms of the regulation of expression of the cyclins by IMP-3, we started by comparing the levels of CCND1 and D3 mRNA and protein in IMP-3 KD cells, compared to control. The short half-life of CCND1 and D3 proteins (30-40 min) allows to perform these studies on endogenous proteins. We have not included CCNG1 in this study, because although its expression is regulated by IMP-3, this atypical cyclin is very stable (with a half-life of over 48h). A clear decrease of the levels of CCND1 and D3 proteins

becomes visible as early as 24-32 h post-transfection of IMP-3 siRNA, whereas the corresponding mRNAs do not vary significantly at these time points (Fig. 1A, B). These results suggest that the expression of the cyclins is initially down-regulated by a post-transcriptional mechanism, which does not depend on the mRNA levels. Next, we have addressed the question of the cyclins' mRNA stability by blocking the transcription in IMP-3 KD versus control cells with 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB). As shown in Fig. 1C, the stability of mRNAs of CCND1 and D3 does not depend on the presence of IMP-3.

As the IMP proteins have been previously shown to control the subcellular transport of some target RNAs, we have studied the efficiency of the nuclear export of the mRNAs of CCND1 and D3 in IMP-3 KD versus control cells, using in situ hybridization. Under both conditions, the CCNs mRNAs were efficiently exported to the cytoplasm, even though in IMP-3 KD cells they appeared to have a more perinuclear localization (Fig. 1D).

The mRNAs of CCND1 and D3 are associated with polyribosomes in IMP-3 KD cells, but their translation is repressed.

To address the translational status of the cyclins' mRNAs in IMP-3 KD cells, we have performed sucrose gradient separation of polyribosomes, followed by RNA isolation from each fraction. Subsequently, we used RT-qPCR to evaluate the relative amounts of the mRNAs of CCND1 and D3 in polyribosomal versus monosomal fractions in IMP-3 KD and control cells. The association of the mRNAs of CCND1 and D3 with polyribosomes did not decrease in the absence of IMP-3 (Fig. 2A). On the contrary, a slight accumulation of these mRNAs in heavy polyribosomal fractions was observed for the mRNAs of CCND1 and D3, but not for the control mRNA of Cyclophilin A. Taken together with the decrease of protein levels of the cyclins in IMP-3 KD cells, these observations suggested that the translation of the mRNAs of CCND1 and D3 can be slowed down in the absence of IMP-3, but this event does not involve the dissociation of the mRNAs from the ribosomes. In agreement with this hypothesis, when we inhibited the protein translation in IMP-3 KD or control cells using the mRNA-ribosome "freezing" drug cycloheximide, we observed a strong decrease of the cyclins in control cells, whereas in IMP-3 KD cells, these was no further decrease of the cyclins' levels when the translation was arrested by the drug. We conclude that in IMP-3 KD cells, the translation of the mRNAs of CCND1 and D3 is already strongly repressed by a mechanism that does not involve mRNA degradation, erroneous mRNA localisation or disassembly of polyribosomes as an initial event.

The expression of CCND1 and D3 in IMP-3 KD cells can be fully restored by inactivating the RISC complex.

The mechanisms of the rapid translational arrest occurring on the mRNAs of CCND1 and D3 in IMP-3 KD cells were unclear. However, the observed phenomenon strongly resembled the translational repression caused by miRNA-dependent recruitment of Argonaute (AGO) proteins and/or of GW182/TNRC6A on target mRNAs (reviewed in (Carthew and Sontheimer 2009)). Multiple publications have earlier identified the mRNAs of CCND1 and D3 as miRNA targets (Jiang, Feng et al. 2009; Bandi and Vassella 2011). In addition, the perinuclear localization of the mRNAs of CCND1 and D3 in IMP-3 KD cells observed in our experiments (Fig. 1D) strongly resembled the typical subcellular localization of activated RISC complexes, as reported in (Stalder, Heusermann et al. 2013). Therefore, we have attempted to simultaneously knock-down key RISC components GW182, AGO1 or AGO2 in our IMP-3 KD cells, and to study the expression levels of CCND1 and D3 proteins. The results presented in Fig. 3A, B clearly show that a KD of GW182 and of AGO2, but not of AGO1, fully restored the levels of CCND1 and D3, even when IMP-3 remained downregulated. The KD of other GW182 family members, TNRC6B and C, which are usually expressed at low levels, did not rescue the expression of the cyclins in IMP-3 KD cells (Fig. S1). We have concluded that IMP-3 could protect the mRNAs of CCND1 and D3 from RNAi in human cancer cells. To find out whether this regulation was direct, we have cloned the 3'UTRs of CCND1 and D3 into the psiCheck-2 luciferase reporter vector, and first studied the roles of AGO1, AGO2, GW182 and IMP-3 in the regulation of the luciferase expression. Consistent with the results observed on endogenous CCND1 and D3, the expression of the luciferase under the control of the 3'UTRs of the cyclins was dramatically down-regulated by a KD of IMP-3, released by a KD of GW182, and slightly increased by a KD of AGO2, but not of AGO1 (Fig. 3C, D). These results clearly indicated that IMP-3 and the RNAi machinery were competing on the 3'UTRs of CCND1 and D3, thus regulating the expression of the cyclins. We have subsequently co-transfected miRNAs let-7g and miR-15, known to regulate CCND1 and D3, respectively, and have further confirmed that while a KD of IMP-3 increased the repressive effect of endogenous or ectopic miRNAs on the cyclins, a KD of GW182 fully reversed this effect.

IMP-3 and GW182/AGO2 bind to and compete within the regions of the 3'UTRs of the cyclins that are critical for their expression.

The IMP proteins do not have a universal binding motif, and were reported to bind to various RNA sequences depending on the cell type and experimental model used in different

studies. Various miRNA-binding sites, both experimentally proven and predicted, are numerous throughout the 3'UTR sequences of CCND1 and D3 (see Introduction for details). Therefore, no prediction was possible as to the functionally important binding sites of IMP-3 protein within these mRNAs. We have decided to identify these regions by a two-step experimental approach (Fig. 4). First, we have synthesized a number of short, partially overlapping mRNA fragments covering the 3'UTRs of the cyclins (Fig. 4AB), biotinylated them, and used them in an in vitro pull-down assay together with total cell extracts from control or IMP-3 KD cells (Fig. 4C-F). In order to test our hypothesis that IMP-3 could hinder the binding of RISC complex to the mRNAs of CCND1 and D3, we were looking for RNA fragments where IMP-3 binding would compete with GW182 and/or AGO2. We used the HuR protein, a very well-characterized post-transcriptional regulator of the expression of CCND1 and D3, as an internal control for the assay, because the binding of HuR to target RNAs depends on the presence of AU-rich sequences, but not on IMP-3 (Brennan and Steitz 2001; Lal, Mazan-Mamczarz et al. 2004).We were able to identify three fragments within the 3'UTR of CCND1 where the binding of RISC complex components was increased in the absence of IMP-3: fragments 3, 6, and 11. In the case of CCND3, IMP-3 competed with GW182 and AGO2 within fragment 3 of 3'UTR.

In the second step of the assay, we attempted to evaluate the functional importance of the identified binding regions of IMP-3 within the 3'UTRs of CCND1 and D3, where it competed with RISC in vitro. To this end, we have transfected the relevant RNA fragments, or the flanking regions of 3'UTRs, where no competition between IMP-3 and RISC had been observed, and quantified the expression of CCND1 and D3 proteins under the control of IMP-3 KD conditions (Fig. 4G-J). If our hypothesis was correct and the depletion of IMP-3 led to excessive presence of RISC on the identified regions of the 3'UTRs of the cyclins, and to translational repression, then the transfection of the corresponding RNA fragments in molar excess should lead to the binding of RISC to the transfected fragments, and to a complete of partial release of the repression. These were the results we have observed in at least three independent experiments (an example of each experiments is shown on the left, and a quantification of three experiments is shown on the right). The transfection of the relevant RNA fragments, identified in Fig. 4C-F, led to a partial (Fig. 4 G, H) or complete (Fig. 4I, J) release of the expression of the cyclins, even in IMP-3 KD cells. Moreover, even in control cells, the transfection of these fragments led to a slight increase of the level of CCND1 and D3, respectively. Therefore, we have experimentally proven that IMP-3 can protect the

mRNAs of CCND1 and D3 from RISC-induced translational repression, and have identified the regions of 3'UTRs targeted by these regulatory mechanisms.

The expression of CCND1 and D3 is co-regulated by IMP-3 protein partners.

A competition between IMP-3 and miRNA-guided GW182 or AGO2 was a novel finding. However, IMP proteins interact with a large amount of other proteins in the cell, both RNA-binding and non-RNA binding (Fig. 5A and (Boudoukha, Cuvellier et al. 2010; Rivera Vargas, Boudoukha et al. 2014)). Thus, it was interesting to determine the exact composition of the IMP-3 subcomplex that was functionally relevant to this new regulatory mechanism. In our previous work, we have identified IMP-3 partners that were important or not for the expression of CCND1, D3, and G1 (Rivera Vargas, Boudoukha et al. 2014). Among them, PTBP1 was a direct IMP-3 partner, and ILF3, HuR, and HNRNPA2B1 depend on the presence of RNA to co-precipitate with IMP-3 (Fig. 5B). Now, we have systematically tested for the involvement of these proteins in competition with GW182. IMP-3 partners PTBP1/HNRNPI and ILF3 were shown to be necessary for the expression of CCND1 and D3, and their KD was compensated by a simultaneous KD of GW182 (Fig. 5CD). ILF3 gene gives rise to two distinct RNA-binding proteins, NF110 and NF90, which are a results of alternative splicing and do not have similar functions in translational regulation (Castella, Bernard et al. 2015). Therefore, we have used isoform-specific siRNAs and have identified the known regulator of cell growth NF90, but not NF110, as the partner of IMP-3 that regulates the expression of CCND1 and D3 in GW182-dependent manner (Fig. 5EF). HuR/ELAVL1 interacts with IMP-3 in an RNA-dependent manner, and regulates the expression of CCND1, but not CCND3 (Fig. 5G). However, the majority of IMP-3 interacting RNA-binding proteins, such as HNRNPA2B1, do not regulate the expression of the cyclins (Fig. 5H and data not shown). These latter results have highlighted the specificity of our experiments and confirmed the existence of a distinct specific subcomplex of IMP-3 that contains PTBP1 and ILF3/NF90, and is critical for the protection of the mRNAs of CCND1 and D3 from RNAi (Fig. 5I).

IMP proteins are known to interact with multiple other proteins and to regulate the fate of numerous mRNAs, most frequently by regulation of mRNA stability, but also by impacting on such diverse processes as mRNA translation, mRNA subcellular localization, and others (Nielsen, Christiansen et al. 1999; Nielsen, Nielsen et al. 2001; Nielsen, Adolph et al. 2003; Oleynikov and Singer 2003; Jonson, Vikesaa et al. 2007; Bell, Wachter et al. 2012; Jonson, Christiansen et al. 2014). These observations suggest the existence of multiple, functionally diverse IMP-dependent RNP complexes, and highlight the necessity to study these complexes

individually and target-wise. We have recently demonstrated that the mRNAs of CCNs D1, D3, and G1 are direct and functionally important targets of IMP-3, as these mRNAs were found within IMP-3 RNP complexes, and in IMP-3 KD cells the expression of these cyclins on protein level was sharply down-regulated. The proliferation of IMP-3 KD cells was arrested due to accumulation of the cells in G1 phase of the cell cycle. This function is specific to IMP-3, and is not al all shared by IMP-1 and IMP-2 (Rivera Vargas, Boudoukha et al. 2014). Now, we have identified the molecular mechanisms of this regulation, which does not depend on regulation of mRNA stability, but involves miRNA and RISC-dependent, reversible translational arrest in the absence of IMP-3 and/or its partners PTBP1 and ILF3/NF90. This ability to bind the cyclins mRNAs and to protect them from RISC is a novel function for a member of the IMP family, showing an important function for IMP-3 in rapid, sensitive and reversible cell cycle regulation of human cancer cells.

Materials and methods

Cell Culture, miRNAs, plasmids and constructs

RD embryonic rhabdomyosarcoma (eRMS) were purchased from ATCC and cultured as indicated on the ATCC website. MiRNA precursors were purchased from Life Technologies and transfected at the final concentration of 50 nM using Lipofectamin RNAiMAX (Invitrogen), according to the manufacturer's instructions. FLAG-HA-IMP3 expression construct and stable cell line was described previously (Rivera Vargas, Boudoukha et al. 2014). psiCHECK-2 reporter constuct was purchased from Promega.

RNA interference

Transient transfection of siRNA was performed using Lipofectamin RNAiMAX. 5 x 10^5 cells were plated in 6-well plates, siRNA duplexes were transfected at 20 nM final concentration for 48 h. The efficiency of the siRNA-mediated knockdown of gene expression was evaluated by quantitative reverse transcription PCR (qRT-PCR) and/or western blotting (WB).

Transient transfection of plasmids was performed using Lipofectamin 2000 (Invitrogen), according to the manufacturer's instructions. 2 x 10^5 cells were plated in 6-well plates; 24 h later the cells were transfected with 1 μg of plasmid for 48 h. Efficiency of transfection was evaluated by immunofluorescence (IF) and/or WB.

siRNA target sequences were: IMP-3 ggauucuccuaguagcauucau; IMP-3_2 auggaucauuccucaugua; GW182/TNRC6A gaaaugcucugguccgcuauu; GW182/TNRC6A_2 gcagccuccagcacaaccucuu; TNRC6B caucugggacaaggugauuguagacg; TNRC6C

ggaauggagacacugugaacucagc ; AGO1 ggaguuacuuucauagcauuu; AGO2 gcacggaaguccaucugaa; PTBP1 aacaaugacaagagccgugac; ILF3 ccccagaggacgacaguaaa; ILF3/NF110 gcggauccgacuacaacuacg; ILF3/NF90 cuuccuagagcgucuaaaagu; HuR gaggcuccagucaaaaacca; HNRNPA2B1 ccaggggcucauguaacugu; Control siRNA uagcaaugacgaaugcgua.

Luciferase reporter assays

The 3'UTR of human CCND1 or CCND3 mRNA were cloned into a psiCHECK2-reporter vector (Promega) downstream of the reporter gene (Renilla luciferase). HeLa cells were seeded at 20 000 cells per well in a 96-well plate. 24h after, 10 ng of psiCHECK2-E2F5-3'UTR was co-transfected with 50 nM of miRNA mimic and/or 20 nM siRNA. Co-transfection was performed with Lipofectamine 2000 (Life Technologies). 48h after transfection, the relative levels of Renilla versus Firefly luciferase activity (control of transfection efficiency) were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luminescence signal was quantified on a Mithras LB 940 Multilabel Reader and analyzed with Mikrowin software (Berthold Technologies).

Antibodies

The following antibodies were used for WB. IMP-3 (N-19) sc-47893, CCND1 (DCS-6) sc-20044, CCND3 (D-7) sc-6283, PTBP1/HNRNPI sc-16547, ILF3 sc-136197 and HuR sc-5261 were obtained from Santa Cruz. GW182 rabbit polyclonal (A302-329A) was obtained from Bethyl Laboratories. Rat anti-AGO1 (SAB4200084), AGO2 (SAB4200085), actin (A5441, clone AC-15), anti-goat IgG (I9140) anti-rabbit IgG (A0545) and anti-mouse IgG (I8765) were purchased from Sigma Aldrich.

Quantitative real-time RT-PCR

The qRT-PCR primers for the detection of IMP-3, CCND1, CCND3, Cyclo A were as described in (Boudoukha, Cuvellier et al. 2010; Rivera Vargas, Boudoukha et al. 2014). qRT-PCR was performed using a Light Cycler (Roche).

Cell lysis and sucrose gradients:

RIPA lysis for WB: The cell pellets were incubated in 5 volumes of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% Na Deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitors] for 15 min on ice followed by sonication for 7.5 min (30 sec ON, 1min OFF) at high intensity. After centrifugation at 16,000 ***g*** for 15 min at 4°C, the supernatant was recovered for WB analysis.

Total lysate from RD cells ($3x10^7$ cells) was prepared as described in (Nielsen, Christiansen et al. 1999), treated or not with 10 mM puromycin for 15 min at 37°C, and applied to a 21 to 47% sucrose gradient in 20 mM Tris-HCl (pH 8.0); 140 mM KCl; 5 mM $MgCl_2$. Centrifugation was carried out at 40,000 rpm for 2h15min using a Beckman SW41 rotor. 0.8-ml fractions were collected, adsorbance at 260 nm was measured, and each fraction was ethanol-precipitated, treated with DNAse (Promega), and RNA was extracted with phenol-chloroform and used for qRT-PCR analysis.

Immunoprecipitation of IMP-3 complexes

Protein complexes were immunoprecipitated from whole cell extracts (lysis buffer: 10% glycerol, 20mM Tris-HCl, pH 8, 0.2 mM EDTA, 0,1% NP-40, 0.5 M KCl, protease inhibitors (Complete, Roche)), pre-cleared for 1h with protein A/G-agarose (Thermo Scientific), treated or not with 1 mg/ml protease-free RNAse A (Roche), using IMP-3 antibody (sc-47893, Santa Cruz) or goat IgG (Sigma) and protein A/G-agarose. Complexes were separated on a 4-12% polyacrylamide gel (Invitrogen), and stained using the Silver Quest kit from Invitrogen, according to manufacturer's instructions. Mass-spectrometry identification of proteins was carried out by Dr. R.Tomaino, Harvard Medical School.

Drug treatment

DRB treatment: 4×10^5 RD Cells were plated in 6-well plates and transfected with control or IMP-3 siRNAs; DRB (Sigma D1916) was added to a final concentration of 100 μM, and the cells were collected at indicated time points.

Cycloheximide Treatment: 4×10^5 RD Cells were plated in 6-well plates and transfected with control or IMP-3 siRNAs; 48 h later, cycloheximide (Sigma C 19881G) was added to a final concentration of 20 μg/ml for 2h.

In Situ hybridization

Probes used were:

CCND1 sense, TAATACGACTCACTATAGGGAGACCCTCGGTGTCCTACTTCAA;
CCND1 antisense, ATTTAGGTGACACTATAGGGGATGGTCTCCTTCATCT;
CCND3 sense, TAATACGACTCACTATAGGGAGATGGATGCTGGAGGTATGTGA
CCND3 antisense, ATTTAGGTGACACTATAGAATGAAGGCCAGGAAATCA

Cells were seeded on poly-Lysine-coated glass cover slides and transfected as previously described. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, washed with PBS, incubated with 0.3% H_2O_2 dissolved in methanol for 30 min at RT and washed with PBS. Afterwards cells were incubated with prehybridization solution (50% formamide, 2X SSC, 10 mM Na_2HPO_4) for 90°C at RT in a humidified chamber followed by

incubation in hybridization solution (1.5 µl salmon sperm DNA, 1.5 µl tRNA and 200-500 ng of DIG-labelled mRNA probes pre-heated for 2 min at 90°C and placed on ice for 2 min, then mixed with 15 µl of 60% formamide, 10 mM Na_2HPO_4 and 15 µl of 20% dextran sulphate, 4X SSC, 0.4% BSA) in a humidified chamber at 37°C overnight. Cells were washed twice with 2 ml of prehybridization solution at 37°C for 30 min, then twice with 2X SSC, 0.1%Triton at 37°-50°C for 5 min, twice with 1X SSC, 0.1%Triton at 37°C for 5 min, and 5 times with PBS-Tween at 37°C for 5 min. Cells were incubated for 1 h in blocking buffer (2% sheep serum, 2 mg/ml BSA, in PBS-Tween) at RT followed by incubation with anti-DIG-biotin antibody (Roche) (diluted 1:500 in blocking buffer) overnight in a humidified chamber at 4°C. To visualize the CCND1 and D3 mRNAs, the TSA BIOTIN SYSTEM (Perkin Elmer) and the Enhanced Liquid Substrate System (Sigma 3,3-Diaminobenzidine) were used according to the manufacturer's instructions.

Protein pull-down by biotinylated RNA

RT reactions were performed using The SuperScript® III First-Strand Synthesis System (Life Technologies) and total RNA from RD cells. Phusion™ High-Fidelity DNA Polymerase Kit for PCR (Thermo Scientific) was used for PCR amplification of desired fragments. All Forward primers had a T7 promoter sequence : TAATACGACTCACTATAGGGAGA. The primers were:

CCND1: NM_053056.2			
Name	Region	Sequence	Location
1 D1 F	3'UTR	ggacgtggacatctgagggc	1082
1 D1 R	3'UTR	ctcccccaccgctcagggtt	1362
2 D1 F	3'UTR	taaccctgagcggtgggggga	1342
2 D1 R	3'UTR	gctttatcaggaaaagcaca	1622
3 D1 F	3'UTR	ttgtgcttttcctgataaag	1602
3 D1 R	3'UTR	tgctacgctggctggtgccc	1882
4 D1 F	3'UTR	cgggcaccagccagcgtagc	1862
4 D1 R	3'UTR	tatttcctacacctattgga	2142
5 D1 F	3'UTR	ccaataggtgtaggaaatag	2122
5 D1 R	3'UTR	actttcaaacaccagttggc	2402
6 D1 F	3'UTR	tgccaactggtgtttgaaag	2382
6 D1 R	3'UTR	aaaataaactgtattaaatc	2662
7 D1 F	3'UTR	agatttaatacagtttattt	2642
7 D1 R	3'UTR	cacttcctaaataaaaatta	2922
8 D1 F	3'UTR	gtaatttttatttaggaagt	2902
8 D1 R	3'UTR	acatggcagtatatgacaca	3182
9 D1 F	3'UTR	caatgtcatatactgccatg	3162

9 D1 R	3'UTR	tctctggggacaccggcgcg	3457
10 D1 F	3'UTR	ccgcgccggtgtccccagag	3437
10 D1 R	3'UTR	aaacagaacactagtacata	3732
11 D1 F	3'UTR	ttatgtactagtgttctgtt	3712
11 D1 R	3'UTR	tggttcagacagacgccgca	4007
12 D1 F	3'UTR	tgcggcgtctgtctgaacca	3987
12 D1 R	3'UTR	ttaccagttttatttctaga	4282
CCND3: NM_001760.3			
Name	Region	Sequence	Location
6 D3 F	3'UTR	gccctctggagtggccacta	1055
6 D3 R	3'UTR	tcccatcagcctggcccacc	1320
7 D3 F	3'UTR	gccaggctgatgggacagaa	1305
7 D3 R	3'UTR	tctaggagcagctgtcagca	1570
3 D3 F	3'UTR	acagctgctcctagagggag	1555
3 D3 R	3'UTR	tatagcagctccttggccac	1820
4 D3 F	3'UTR	caaggagctgctatagcctg	1805
4 D3 R	3'UTR	tttttccaagaagccaaagc	2055

MEGAscript® T7 Transcription Kit (Life Technologies) was for RNA in vitro transcription, and RNA 3' End Biotinylation Kit (Pierce) for biotinylation of RNA fragments, all of the above according to the manufacturers' instructions.

Biotin pull down assay was performed as follows (our original protocol):

Necessary reagents :

RNaseOUT™ Recombinant Ribonuclease Inhibitor.

Complete Protease Inhibitor Cocktail (Roche).

Pierce IP Lysis Buffer is composed of 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol.

Cell lysis in light conditions : Incubate the cell pellet with Pierce lysis buffer + protease inhibitor for 15 min on ice. Up and Down for 3 times at the beginning of incubation. Centrifugate (15 min × 13,200 rpm × 4°C) and collect the supernatant (protein lysate).

TENT buffer (50 mM Tris-Cl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% Triton X-100).

1) Pull down reaction mix :

RNaseOUT (40U/ µL)	2.5 µL
Protease inhibitor cocktail 50x	1 µL
Biotinylated RNA probe	10 µL
Protein lysate	40 µg
TENT 2x	25 µL
H2O	7.5 µL
Final volume	50 µL

Incubate pull down reaction mix on a wheel 30 min × 20 rpm at RT

2) Beads washing and distibution.

Utilize 30 μL of Streptavidin-Agarose Beads per reaction. Wash and centrifugate (2 min × 2000 rpm × 4°C) Beads briefly 3 times with 1 ml of TENT 1X. After the last wash add 1 ml TENT 1X + Protease Inhibitor + RNaseOUT 1000U/ml and distribute them between the necessary amount of tubes, centrifugate (2 min × 2000 rpm × 4°C), remove the supernatant and add pull-down reaction mix.

3) Incubate pull-down reaction mix + beads on a wheel 30 min × 20 rpm × RT. Wash beads 3 times × 10 min × 20 rpm in 500 μL PBS.

Detect proteins by standard WB technique.

Conflict of interest

The authors declare that there are no competing financial interests related to this study.

Acknowledgements

The authors thank Drs. A Harel-Bellan, F. Dautry and Y. Vassetzky for helpful discussions. This study was a PhD project of E. Deforzh, based on previous results obtained by T. Rivera Vargas, both studies performed under the direction of A. Polesskaya.

Legends to Figures

Figure 1. *The expression of CCND1 and D3 is regulated on post-transcriptional level in IMP-3 KD cells.* A, B. Kinetics of inhibition of CCND1 and D3 in RD cells transfected with the indicated siRNAs and collected at 8, 24, 32, 48, 72, and 100 h post-transfection. A, protein levels (western blot), B, mRNA levels (RT-qPCR). C. Relative rate of mRNA degradation in control versus IMP-3 KD cells under the conditions of general transcription inhibition by DRB. D. In situ hybridization showing the subcellular localization of CCND1 and D3 mRNAs in RD cells transfected with the indicated siRNAs.

Figure 2. *The mRNAs of CCND1 and D3 are associated with polyribosomes in IMP-3 KD cells, but their translation is repressed*. A. Sucrose gradient fractionation of cytoplasmic

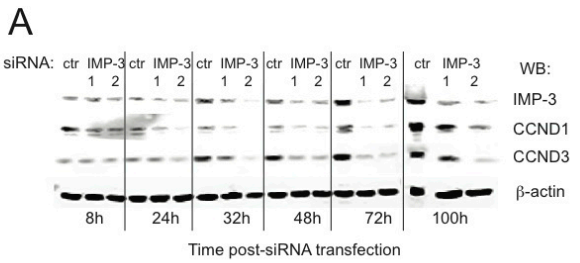
lysates of RD cells transfected for 48h with control siRNA or with two distinct anti-IMP-3 siRNAs. The mRNAs of CCND1 and D3 and cyclophilin A (cycloA) were quantified in each fraction using RT-qPCR. B, C. RD cells were transfected with control or IMP-3 siRNA, and treated at 48h post-transfection with 20 mg/ml cycloheximide or ethanol for 2h. Expression of CCN D1 and D3 was evaluated by WB and quantified using ChemiCapt (Vilber Lourmat). Shown are typical results (B) or a mean (A, C) of 3 independent experiments.

Figure 3. *The expression of CCND1 and D3 in IMP-3 KD cells can be fully restored by inactivating the RISC complex.* A. A WB analysis shows a rescue of CCND1 and D3 expression in IMP-3 KD cells when GW182 is knocked-down. B. The expression of CCND1 and D3 in IMP-3 cells can be rescued by a KD of AGO2, but not of AGO1 (lanes 1-5). In control cells, the KD of AGO1, AGO2 or GW182 has no effect on the levels of CCND1 and D3 (lanes 5-9). C-E. The regulation of luciferase expression (psiCHECK-2 reporter vector) under the control of 3'UTRs of CCND1 (C, E) and CCND3 (D, F) depends on IMP-3, GW182, AGO2 and miRNAs let-7g (CCND1) and miR-15 (CCND3). The luciferase expression of each vector co-transfected with control siRNA used was set to 1 and used for normalization of all the respective assays. Error bars represent SEM from 3 independent experiments. p values (above each bar) were calculated using a paired t test comparison of the condition with the control siRNA condition: *$p < .05$; **$p < .01$; ***$p < .001$.

Figure 4. *IMP-3 and GW182/AGO2 bind to and compete within the regions of the 3'UTRs of the cyclins that are critical for their expression.* A, B. A schematic representation of the 3'UTRs of CCND1 (A) and D3 (B) with the coordinates of the fragments used for pull-down experiments. C-E. Indicated fragments of the 3'UTRs of CCND1 and D3 were transcribed in vitro, biotinylated, and incubated with total cell extracts from control of IMP-3 KD cells. The proteins bound to the RNA fragments were revealed using western blot. G-H. RNA fragments characterized by IMP-3-RISC competitive binding, or control fragments form the CCNs 3'UTRs (identified in C-E) were transfected into control or IMP-3 KD RD cells, and the expression of CCND1 and D3 proteins was quantified by western blot in three independent experiments. Left, a representative experiment out of three, right, quantification of three experiments.

Figure 5. *The expression of CCND1 and D3 is co-regulated by IMP-3 protein partners.* A. Silver staining of IMP-3 protein complexes immunopurified from RD cells, untreated or

treated with RNase A. Mock, IgG. B. Co-immunoprecipitation analysis of endogenous IMP-3 interaction with the indicated partners. C-G. A WB analysis shows the rescue levels of CCND1 and D3 expression in cells depleted of IMP-3 protein partners PTBP1 (C), ILF3 (D), ILF3/NF90 (E), ILF3/NF110 (F), and HuR (G) when GW182 (lower band on the WB) is knocked-down. H. An IMP-3 protein partner HNRNPA2B1 does not influence the expression of CCND1 and D3. I. A model of action for IMP-3/NF90/PTBP1 complex in the inhibition of RISC-dependent translational repression. Antagonistic interactions between IMP-3/NF90/PTBP1 and RISC complexes: IMP-3 complex can interfere with miRNA function via competition for the same binding site (on the right). In the context of non-overlapping binding sites IMP-3 complex can change local mRNA structure and prevent RISC binding (on the left).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CCND3
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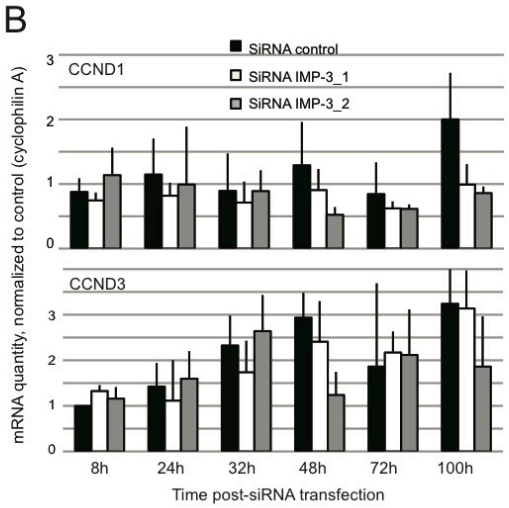
24h
32h
48h
72h
100h
Time post-siRNA transfection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SiRNA IMP-3_2
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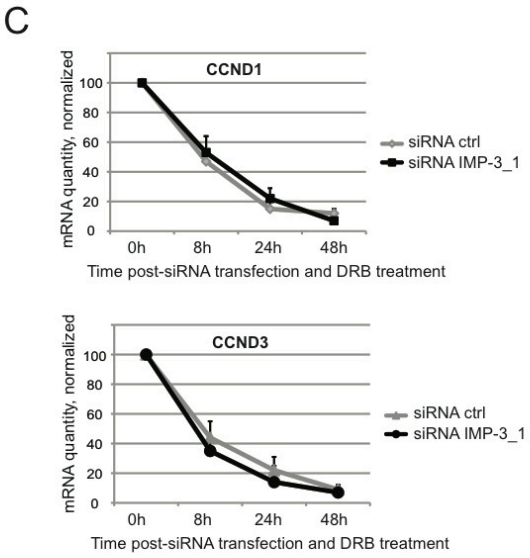
8h
24h
32h
48h
72h
100h
Time post-siRNA transfection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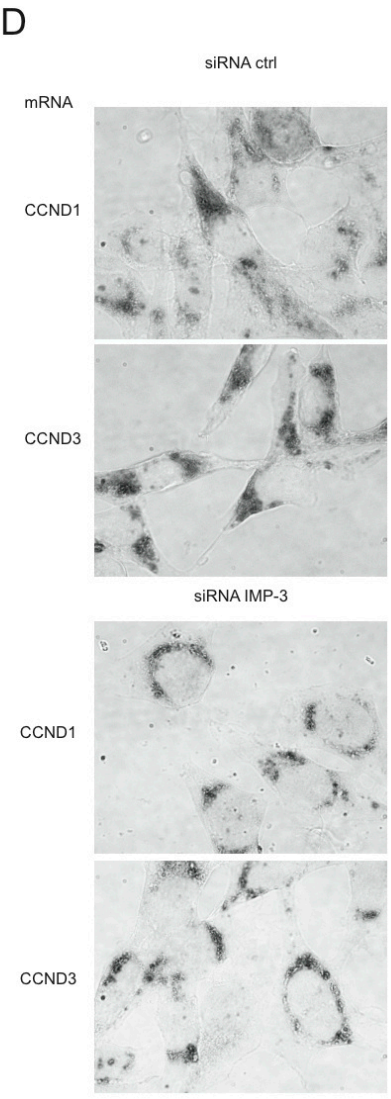
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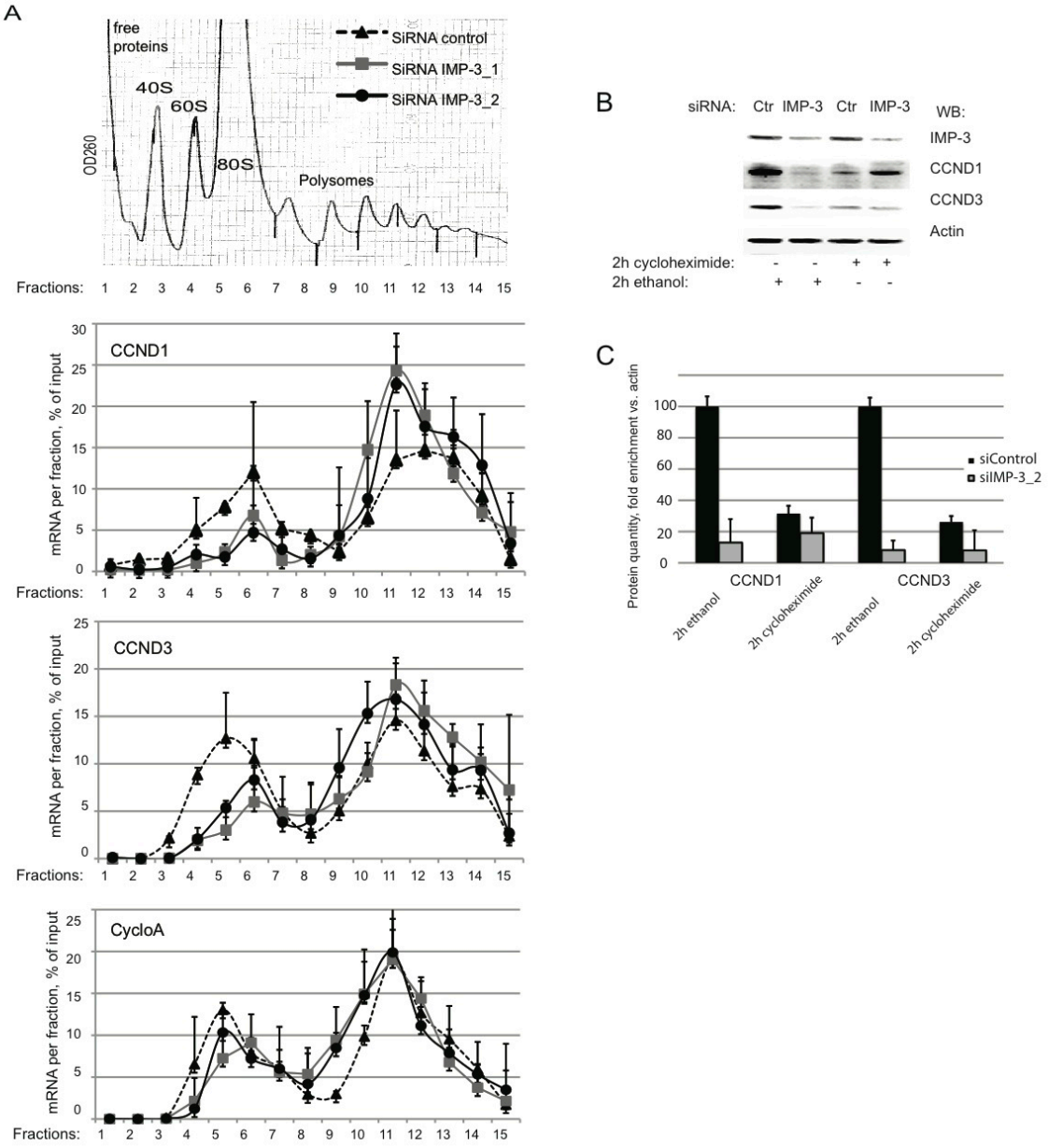
CCND3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80S
Polysomes
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SiRNA control
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SiRNA IMP-3_2
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CCND1
mRNA per fraction, % of input
CCND3
CycloA
B
siRNA: Ctr IMP-3 Ctr IMP-3
WB:
IMP-3
CCND1
CCND3
Actin
2h cycloheximide: - - + +
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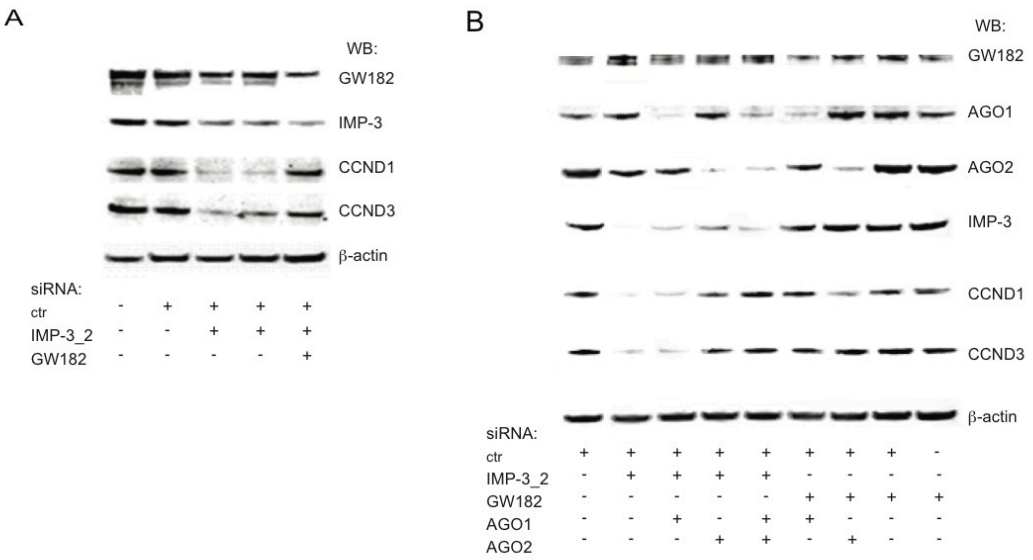
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AGO2
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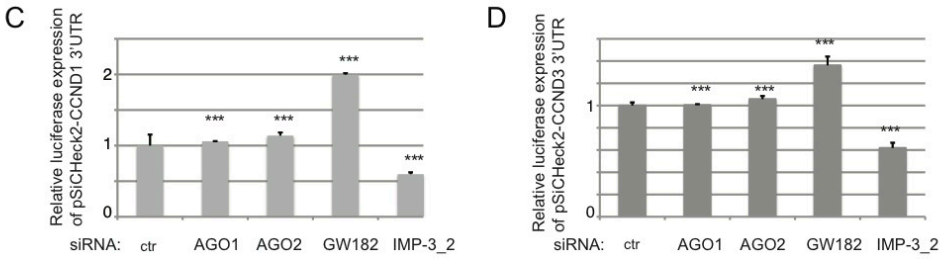
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0
***
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***
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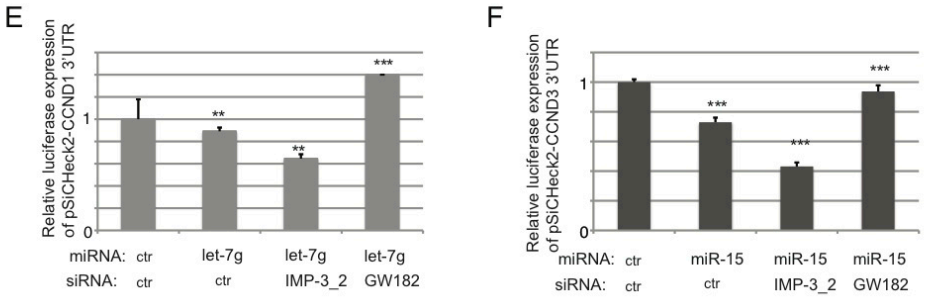
E
Relative luciferase expression of pSiCHeck2-CCND1 3'UTR
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0
**
***
miRNA: ctr let-7g let-7g let-7g
siRNA: ctr ctr IMP-3_2 GW182
F
Relative luciferase expression of pSiCHeck2-CCND3 3'UTR
1
0
***
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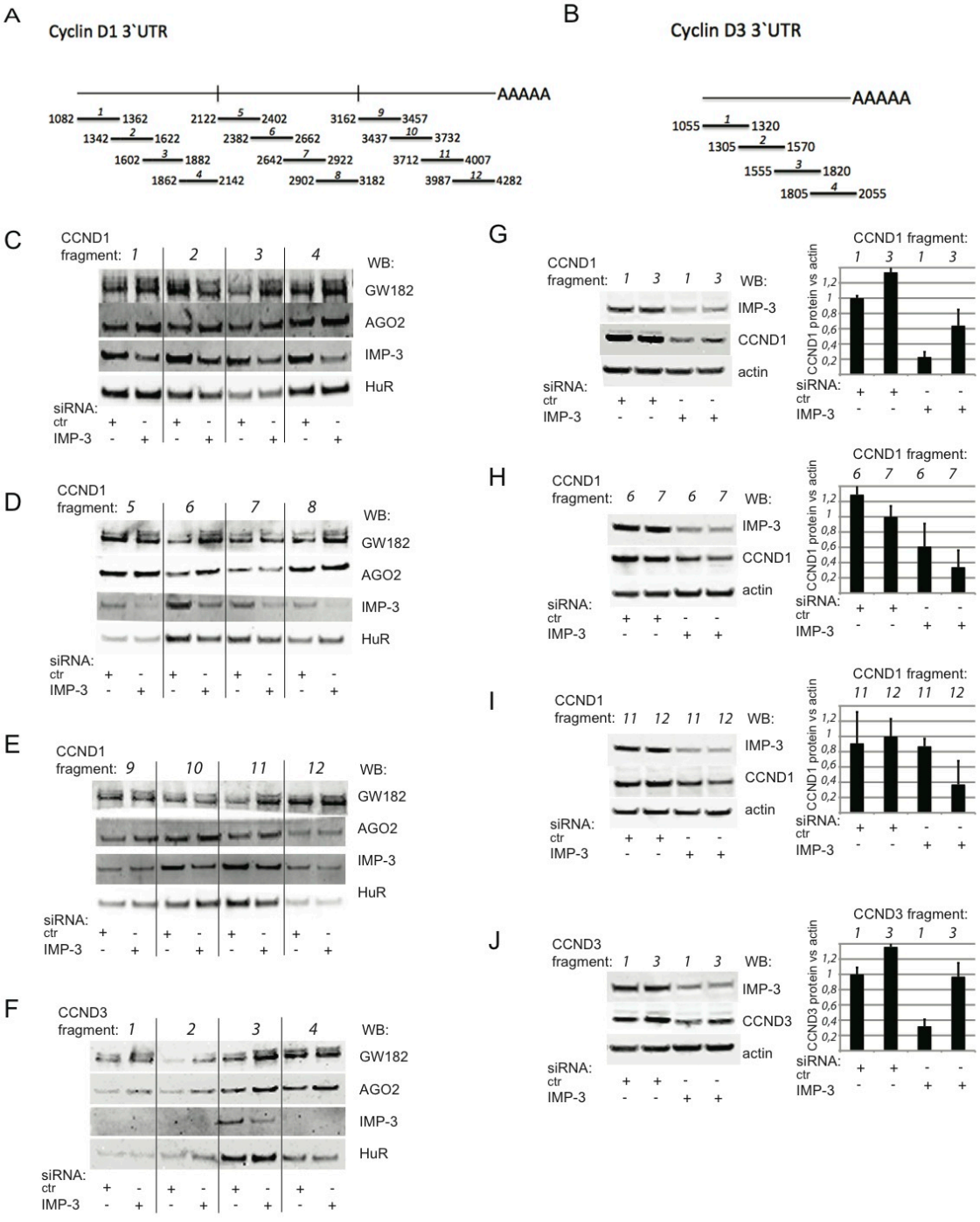
siRNA: ctr ctr IMP-3_2 GW182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3437 10 3732
3712 11 4007
3987 12 4282
B
Cyclin D3 3`UTR
AAAAA
1055 1 1320
1305 2 1570
1555 3 1820
1805 4 2055
C
CCND1 fragment: 1 2 3 4
WB:
GW182
AGO2
IMP-3
HuR
siRNA:
ctr + - + - + - + -
IMP-3 - + - + - + - +
D
CCND1 fragment: 5 6 7 8
WB:
GW182
AGO2
IMP-3
HuR
siRNA:
ctr + - + - + - + -
IMP-3 - + - + - + - +
E
CCND1 fragment: 9 10 11 12
WB:
GW182
AGO2
IMP-3
HuR
siRNA:
ctr + - + - + - + -
IMP-3 - + - + - + - +
F
CCND3 fragment: 1 2 3 4
WB:
GW182
AGO2
IMP-3
HuR
siRNA:
ctr + - + - + - + -
IMP-3 - + - + - + - +
G
CCND1 fragment: 1 3 1 3
WB:
IMP-3
CCND1
actin
siRNA:
ctr + + - -
IMP-3 - - + +
CCND1 fragment: 1 3 1 3
CCND1 protein vs actin
1,2
1
0,8
0,6
0,4
0,2
siRNA:
ctr + + - -
IMP-3 - - + +
H
CCND1 fragment: 6 7 6 7
WB:
IMP-3
CCND1
actin
siRNA:
ctr + + - -
IMP-3 - - + +
CCND1 fragment: 6 7 6 7
CCND1 protein vs actin
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CCND1 fragment: 11 12 11 12
WB:
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CCND1
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siRNA:
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CCND1 protein vs actin
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siRNA:
ctr + + - -
IMP-3 - - + +
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CCND3 fragment: 1 3 1 3
WB:
IMP-3
CCND3
actin
siRNA:
ctr + + - -
IMP-3 - - + +
CCND3 fragment: 1 3 1 3
CCND3 protein vs actin
1,2
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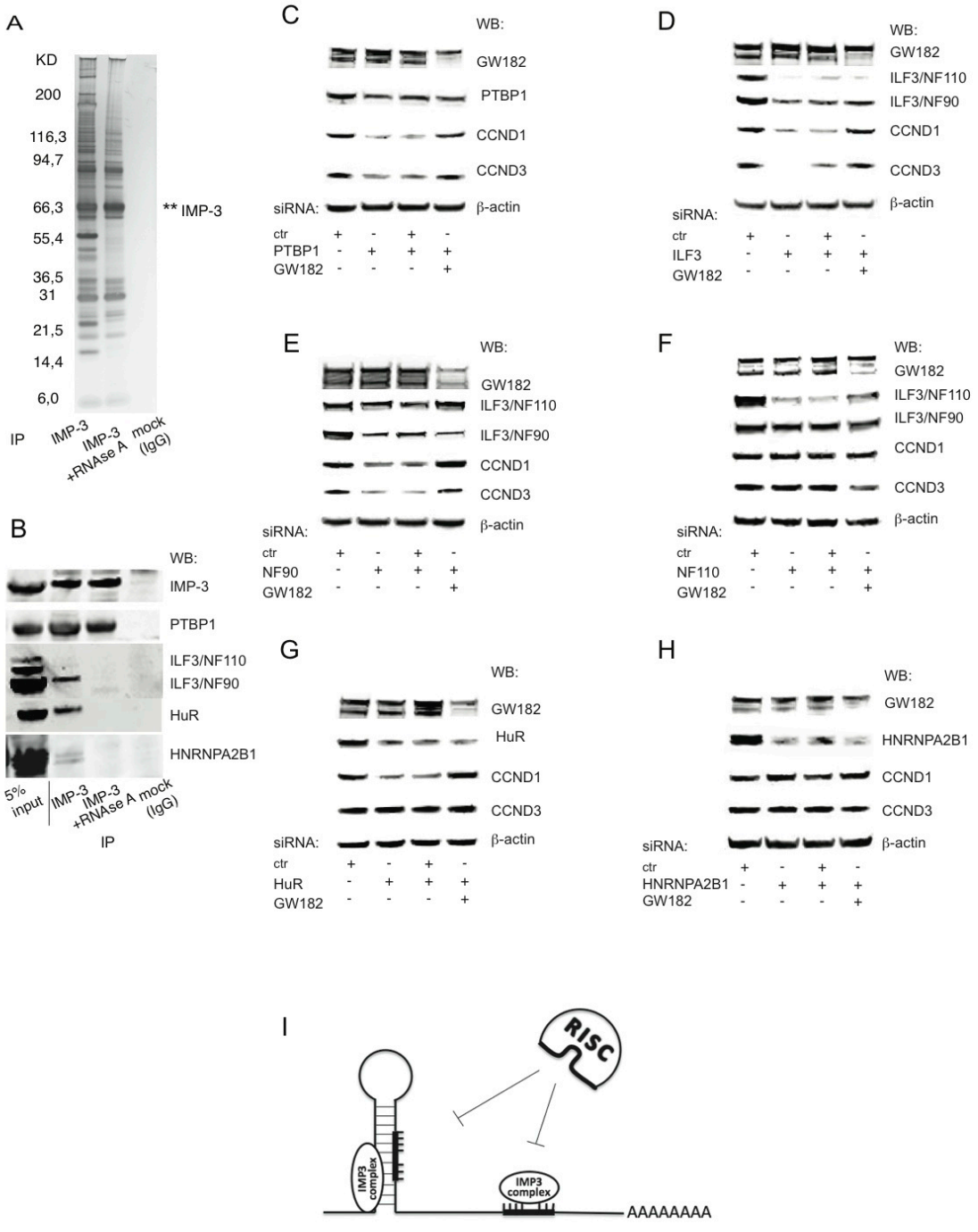
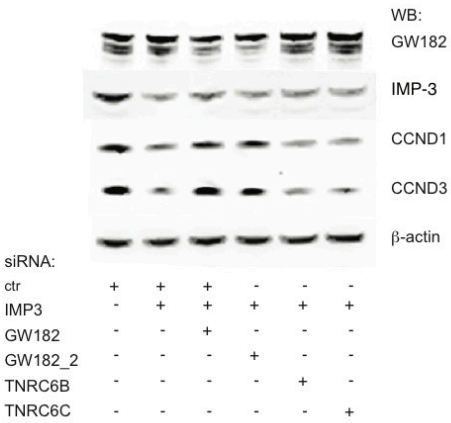
siRNA:
ctr + + - -
IMP-3 - - + +

Deforzh et al., Fig. 5



Deforzh et al., Fig. S1



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Article N°2 (submitted). The WEE1 regulators CPEB1 and miR-15b switch from inhibitors to activators at G2/M.

During my PhD work I contributed to the research project of Irina Groisman, researcher in our laboratory. She demonstrated that a known RNA-binding protein CPEB1 works in cooperation with miR-15b to repress WEE1 mRNA (key mammalian cell cycle regulator) translation during G1 and S-phase. But at G2/M transition CPEB1 and miR-15b switch their activity and promote translation of WEE1 mRNA.
In this project, I have studied the interaction between CPEB1 and miR-15b/RISC. With a help of co-immunoprecipitation experiments I demonstrated that these two complexes can bind to common RNA targets and interact in RNA-dependent manner. I also detected the helicase DDX6 immunoprecipitated with CPEB1 and AGO2/RISC and demonstrated that a KD of DDX6 or using of DDX6 mutant with lost activity disrupt interaction between Ago2/RISC and CPEB1.
These results were included in the following article.

The WEE1 regulators CPEB1 and miR-15b switch from inhibitor to activators at G2/M

Gueorgui Kratassiouk[1,2,3], Linda L. Pritchard[1,2,3], Sylvain Cuvellier[1,2,3,4], Andrii Vislovukh[1,2,3,5], Qingwei Meng[1,2,3,6], Regina Groisman[1,2,3], Cindy Degerny[1,2,3], Evgeny Deforzh[1,2,3], Annick Harel-Bellan[1,2,3,7] & Irina Groisman[1,2,3,7]*

[1]Université Paris Sud, Laboratoire Epigénétique et Cancer, Formation de Recherche en Evolution 3377, F-91191 Gif-Sur-Yvette, France.

[2]Centre National de la Recherche Scientifique (CNRS), F-91191 Gif-Sur-Yvette, France.

[3]Commissariat à l'Energie Atomique (CEA), Saclay, F-91191 Gif-sur-Yvette, France.

[4]Present address: Inserm U1016, Institut Cochin, Département Génétique et Développement, F-75014 Paris, France

[5]Present address: Department of Translation Mechanisms, Institute of Molecular Biology and Genetics, National Academy of Sciences, Kiev, 03680 Ukraine

[6]Present address: The Breast Department of the Third Affiliated Hospital of Harbin Medical University, Harbin, 246 China

[7] Equal contributions.

*To whom correspondence should be addressed: Tel: +33169085376, fax : +33169084712, Email: irina.groisman@cea.fr

Present Address: Irina Groisman, Service de Biologie Intégrative et Génétique Moléculaire (SBIGEM), Commissariat à l'Energie Atomique (CEA), Saclay, F-91191 Gif-sur-Yvette, France.

Running title: CPEB1 and miR-15b control WEE1 expression

Keywords: miR-15b, CPEB1, WEE1, cell cycle, translational regulation, cytoplasmic polyadenylation

Summary

MicroRNAs (miRNAs) in the AGO-containing RISC complex control messenger RNA (mRNA) translation by binding to mRNA 3' untranslated region (3'UTR). The relationship between miRNAs and other regulatory factors that also bind to mRNA 3'UTR, such as CPEB1 (cytoplasmic polyadenylation element-binding protein), remains elusive. We found that both CPEB1 and miR-15b control the expression of WEE1, a key mammalian cell cycle regulator. Together, they repress WEE1 protein expression during G1 and S-phase. Interestingly, the two factors lose their inhibitory activity at the G2/M transition, at the time of the cell cycle when WEE1 expression is maximal, and, moreover, rather activate *WEE1* translation in a synergistic manner. Our data show that translational regulation by RISC and CPEB1 is essential in cell cycle control and, most importantly, is coordinated, and can be switched from inhibition to activation during the cell cycle.

Introduction

The importance of 3'UTR (3' untranslated region) in the control of translation and mRNA stability has been known for many years. One of the best-studied RNA regulatory sequences located in 3'UTRs is the CPE (cytoplasmic polyadenylation element). CPE is bound by CPEB1 (cytoplasmic polyadenylation element binding protein), a four-member protein family.

CPEB1 was initially found to be important for *Xenopus* oocyte maturation [1]. It was later seen to be involved in learning and memory [2, 3] and in the regulation of the mammalian cell cycle [4-7]. During *Xenopus* oocyte maturation, CPEB1 controls meiosis progression from prophase I to metaphase II, triggering tightly controlled waves of polyadenylation at various phases of meiosis [8], as well as during the embryonic cell-cycle [5]. In mammals, CPEB1 is also implicated in senescence [4, 6] and in controlling the translation of proteins involved in cell-cycle checkpoints [7]. CPEB1 is a conserved, sequence-specific RNA-binding protein containing a zinc finger and two RNA recognition motifs (RRMs) [1, 8-10]. *Xenopus* studies show that CPEB1 can both promote and inhibit RNA translation by respectively elongating or shortening mRNA poly(A) tails since CPEB1 recruits adenylating and/or de-adenylating protein complexes. This dual action of CPEB1 changes over the course of the cell cycle, depending on CPEB1 post-transcriptional modifications and on the number and location of the CPEs to which CPEB1 binds. The CPEB1-containing complex in Xenopus includes: symplekin, which may be a platform protein upon which multi-component complexes are assembled; poly(A) ribonuclease (PARN), which is a deadenylating enzyme, and germ-line-development factor 2 (Gld2), which is a poly(A) polymerase [11, 12]. Induction of cytoplasmic polyadenylation is mediated by

activation of the serine/threonine kinase Aurora A/Eg2, possibly via repression of glycogen synthase kinase 3 [10, 13]. When phosphorylated on either S174 or T171 (which is species-dependent), CPEB1 promotes polyadenylation by stimulating the activity of Gld-2 [11], an atypical poly(A) polymerase [14]. The newly elongated tail is then bound by the poly(A)-binding protein (PABP), which promotes translation by facilitating assembly of the eIF4F initiation complex [15].

The miRNA (microRNA) system is another well-known regulator of mRNA translation. MicroRNAs are single-stranded RNA molecules of about 21-23 nucleotides in length, which are transcribed as 70-90 nt precursors and further processed to short double-stranded sequences by the endonuclease DICER. MiRNAs regulate gene expression by forming miRNA-induced silencing complexes (miRISCs). MiRISCs inhibit translation by binding through the microRNA strand to imperfectly matched sequences in the 3'UTR of target mRNAs. The MiRNA mode of action is a much-debated issue. However, there are exist experimental proofs supporting collaboration between the RISC complex, which contains the proteins argonaute 1 & 2 (AGO1 and AGO2), and the deadenylation complex [16, 17]. The mRNA targets of miRNAs are frequently subject to deadenylation [18, 19], further supporting the idea that the length of the poly(A) tail is a key element in the control of translation by miRNAs. Thus, both miRNAs and CPEB1 control the length of mRNA poly(A) tails, raising the possibility that they may cooperate to regulate common targets.

CPEB1 and RISC complexes have been found in processing bodies (P-bodies), which are sites of mRNA degradation and storage, as well as in stress granules, where translation initiation complexes are stored under various stress conditions. It is worth mentioning that DDX6 (rck/p54), a DEAD-box helicase that interacts with AGO1 and AGO2 in cells and is

essential in P-bodies [20] and stress granules, associates with CPEB1 in both *Spisula* (clam p47) and *Xenopus* [21] and could therefore constitute a link between CPEB1 and the RISC complex.

All these observations suggest that CPEB1 and RISC cooperate. Here, we address this issue by investigating the functional interaction between CPEB1 and a nearby miRNA-binding site on the 3'UTR of *WEE1* mRNA. WEE1 is a kinase component of the G2/M cell-cycle checkpoint. WEE1 determines the time of entry into mitosis, thereby influencing the size of daughter cells. Loss of WEE1 results in smaller than normal daughter cells, due to premature cell division. Although WEE1 kinase has long been characterized as a key inhibitor of cyclin-dependent kinase 1 (Cdk1) and of mitotic entry in eukaryotes, the regulation of WEE1 expression and activity is still not fully understood. WEE1 is regulated at the post-translational level by phosphorylation [22]. During *Xenopus* oocyte maturation, *WEE1* mRNA translation is regulated by a CPE sequence located in its 3'UTR [23]. *WEE1* mRNA CPE is conserved in the human. In addition, the 3'UTR of human *WEE1* mRNA contains a miR-15b binding site, and WEE1 is one of the high-score predicted targets for miR-15b (using two algorithms, Targetscan and Microcosm). And it was indeed proved that WEE1 could be a target of miR-15b under a certain physiological condition [24]. We therefore explored how each of these two elements of *WEE1* 3'UTR, the CPE and miR-15b binding sites, function during the mammalian cell cycle.

We found that the CPE and miR-15b sequences in WEE1 3'UTR are both important for WEE1 expression, being independent inhibitors of *WEE1* 3'UTR-dependent translation in G1 and S cells. CPEB1 and AGO2 (RISC) complexes interacted in an RNA- and DDX6

(rck/p54)-dependent manner. Importantly, we observed that the inhibitory effects of miR-15/RISC and CPEB1 were abolished during G2/M transition. Moreover, the two sequences together had an activating effect at this stage of the cell cycle when WEE1 protein is maximal, as documented by luciferase assays or polysomal profiles of reporter mRNAs, as well by analyzing WEE1 endogenous protein in CPEB1- and/or miR-15b-depleted cells. Our data demonstrate that WEE1 expression is regulated at the translational level in a cell-cycle-dependent manner. Moreover, they show that the regulatory activities of two RNA binding complexes are modified in a coordinated manner, switching from being activators to inhibitors during the cell cycle.

Results

WEE1 is regulated at the prost-transcriptional level in a 3'UTR-dependent manner

WEE1 expression is regulated at the level of translation during *Xenopus* oocyte maturation [23]. In HeLa cells synchronized by a double thymidine block, WEE1 protein expression was regulated in a cell-cycle dependent manner (Figure 1A, B), increasing at the G2/M transition. This regulation occurred mostly at the post-transcriptional level, since *WEE1* mRNA did not vary significantly during cell-cycle progression (Figure 1C).

In *Xenopus* oocyte, *Wee1* 3'UTR includes three CPEs, and *Wee1* mRNA translation is controlled by CPEB1 (Charlesworth et al., 2000). Human *WEE1* mRNA contains 2 CPEs. Interestingly, an miR-15b binding site is located 180 nucleotides upstream of the first CPE-site (Figure 1D). In order to explore the role of these elements in WEE1 regulation, we generated *Renilla*-luciferase reporter constructs containing, either wild type or mutated *WEE1* 3'UTR in the first CPE or the miR-15b sites, alone or in combination (Figure 1D). We focused our attention on the first CPE, because of its close location to the miR-15b site. In HeLa cells, reporter activity was stimulated by mutations in the CPE or miR-15b site (Figure 1E). Mutating the two sites led to higher expression than mutating each one alone, and the effects were additive. These data suggest that both CPEB1 and miR-15b control WEE1 expression in an independent manner. To further confirm this hypothesis, we used CPEB1 [25] and/or miR-15b [26] loss-of-function assays. First, we confirmed the activity of the inhibitors by Western-blot analysis and QRT-PCR, respectively (Figure. 2A, B). Treating the cells with either one of the inhibitors increased the activity of the wild-type *WEE1* 3'UTR reporter, while simultaneous depletion of CPEB1 and inhibition of miR-15b was even more effective (Figure 2C), well correlated with the results obtained by mutating

CPEB1 and miR-15b binding sites (Figure 2C and 1D). It is important to note that depletion of CPEB1 did not affect luciferase activity in the absence of the CPE sequence, nor did inhibition of miR-15b affect luciferase in the absence of the miR-15b binding site (Figure 2C), underscoring the specificity of the inhibition. Thus both CPEB1 and the RISC complexes appear to regulate *WEE1* mRNA through their respective binding sites. This hypothesis was further supported by the subcellular localization of *WEE1* reporter mRNA, CPEB1, and miR-15b (Figure 2D). The three components were observed in dots that are likely to be P-bodies or stress granules, since they contain DDX6/p54 or TIA1 (Figure S1 A, B, C). Some granules containing the reporter mRNA also contained both miR-15b and CPEB1. Mutation of each binding site alone did not reduce the presence of the reporter mRNA in dots containing the other partner, but mutation of the two sites did abolish the recruitment of the reporter mRNA into granules (Figure 2D and S2 A, B).

We next tested whether endogenous WEE1 protein was affected by modulating CPEB1 and/or miR-15b levels. When CPEB1 and/or miR-15b were down-regulated or inhibited (Figure 2A, B, S3A), endogenous WEE1 protein was increased (Figure 2E, S3B). Importantly, depletion of CPEB1 and/or miR-15b had little effect on *WEE1* mRNA levels (Figure S3C). Conversely, overexpressing CPEB1 (Figure 2G) reduced WEE1 protein expression (Figure 2E). Overexpressing miR-15b (Figure 2F) did not impact on WEE1 protein, possibly due to the fact that this miRNA is already highly expressed in HeLa cells.

Interaction between CPEB1 and miR-15b/RISC

Since the CPE and miR-15b binding sites are in close proximity, next we used co-immunoprecipitation experiments to investigate whether CPEB1 and RISC could

physically interact. AGO2 co-immunoprecipitated with overexpressed FLAG-Myc-CPEB1, but this interaction was RNA-dependent (Figure 3A). Conversely, CPEB1 co-immunoprecipitated with exogenous FLAG-HA-AGO2, and this was also dependent on the presence of RNA (Figure 3B). Thus, the two complexes may bind to common mRNA targets.

As previously published [20, 21], we detected the helicase DDX6 in both CPEB1 and AGO2 FLAG-tag immunoprecipitated complexes (Figure 3C, E). Knocking-down DDX6 (with an siRNA) or inhibiting its activity with a helicase-dead transdominant mutant [27] abolished interaction between CPEB1 and AGO2 (Figure 3C-E).

The activity of CPEB1 and miR-15/RISC is coordinated during the cell cycle

WEE1 is regulated in a cell-cycle-dependent manner (Figure 1A), and the activity of CPEB1 is also cell cycle dependent in *Xenopus* embryo, CPEB1 acting alternatively as a repressor and an activator of gene translation (Groisman et al., 2002). We therefore investigated the importance of CPEB1 and miR-15b for WEE1 expression during the cell cycle, after releasing HeLa cells from a double thymidine block (Figure 4A). Surprisingly, we observed that when cells progressed toward G2/M, not only the CPE, but also the miR-15b sites lost their inhibitory activity (Figure 4B). Coincidentally, silencing granules that contained *WEE1* mRNA disappeared in G2/M cells (Figure S4A), implying that WEE1 mRNA is released into the cytoplasm and may be actively translated.

Moreover, WEE1 protein expression was maximal upon entering G2/M (Figure 4C). Both sites tend to have an opposite effect, which is to stimulate translation. Note that in this case, the activities of the two sites were synergistic rather than additive, both sites being required

for activation. A more detailed time-course analysis (hourly, beginning 28 hours after transfection, supplementary Figure S4B) showed that during G2/M, the CPE and miR-15b sequences in *WEE1* 3'UTR sequentially lost their inhibitory activity, then, when most of the cells had progressed into G2/M, together they became slightly stimulating for luciferase expression (Figure S4C). When the cells returned to the G0/G1 cell-cycle stage, the inhibitory functions of CPE and miR-15b binding sites were restored (Figure S4C). Thus, CPEB1 and miR-15b seem to lose their inhibitory activity at the G2/M phase of the cell cycle. CPEB1 protein was down regulated during G2/M, but CPEB1 mRNA level was stable (Figure S4D, E), similar to what was observed during *Xenopus* oocyte maturation. MiR-15b, were constitutively expressed throughout the cell cycle (Figure S4F). Importantly, the *WEE1* mRNA level did not change throughout the cell cycle (Figure S4G), as observed earlier (Figure 1C).

Our results support the hypothesis that WEE1 translation is regulated in a cell-cycle-dependent manner. In order to further explore this hypothesis, we monitored WEE1 reporter translation throughout the cell cycle. Active translation of mRNAs can be monitored by their presence in the heavy fractions of a sucrose gradient, which is interpreted as association of mRNA with multiple ribosomes in polysomes. In a population of predominantly G0/G1 cells (83%), WEE1 wild-type 3'UTR was distributed into three peaks, with more than half in the monosomal or light polysomal fractions (Figure 4C, fractions 1 to 7), implying low translational activity. When only the CPE or, alternatively, the miR-15b sequence was mutated, the reporter mRNA shifted toward the heavier polysomal fractions (fractions 5 to 8) (Figure S5A). Mutating both the miR-15b and CPE sites eliminated the two light peaks, and the reporter was then mostly associated with heavy

polysomes (fractions 9 to 12), indicating high translational activity (Figure 4D). In a population with at least 50% of cells in G2/M, wt *WEE1* 3'UTR was mostly associated with the heavy polysomal fractions (fractions 8 to 12). However, when the miR-15b binding site, the CPE site, or both were mutated, the mRNA shifted to the lighter polysomal fractions (fractions 5 to 8) (Figure 4D, and S5A). This result is correlates well with the reduced luciferase activity expressed by the same constructs in G2/M cells (Figure 4B, S4C). Moreover, also in this assay, mutating one of the two sites had the same effect in G2/M cells as mutating both of them (Figure 4D and S5A), implying synergy between the two complexes. The control *GAPDH* mRNA was constitutively associated with heavy polysomal fractions in all samples (Figure 4D and S5B). These data amount to strong support of the idea that CPEB1 and miR-15b activity on WEE1 mRNA depends on the cell cycle, with both regulators being inhibitors in the G1 and/or S phases and activators at G2/M.

This idea was further supported by analysis of WEE1 protein accumulation during the cell cycle, since WEE1 protein was deregulated by CPEB1 and/or miRNA depletion (Figure 5A, B). In control cells, WEE1 protein was low in G1 or S cells (1h post-release) and increased at G2/M (5h post-release), whereas *WEE1* mRNA levels remained quite stable throughout the cell cycle (Figure 5C). When both CPEB1 and miR-15b were depleted, WEE1 protein and, coincidently, Cyclin B1, used here as a cell-cycle marker, were deregulated, showing little variation in the time frame of the experiment (Figure 5A). Most interestingly, when miR-15b, CPEB1, or both were depleted, WEE1 protein was higher in G1/S cells than in control cells (Figure 5B), as expected if the two regulators are inhibitors. In sharp contrast, in G2/M cells (5h post-release) depletion of CPEB1, miR-15b, or both

resulted in reduced WEE1 expression, as expected for activators. Even though Western blot is not a fully quantitative technique, these data clearly indicate a tendency. Thus, the expression of endogenous WEE1 protein appears to be dependent on miR-15b and CPEB1 in G2/M cells, consistent with the data obtained using the WEE1 reporter construct (Figure 4). Depletion of DDX6 also affected the expression of WEE1 and Cyclin B1 (Figure S6). Together, these data indicate that CPEB1 and miR-15b/RISC are required for proper expression of WEE1 protein during the cell cycle.

Since WEE1 regulates the temporality of cell-cycle progression, affecting WEE1 expression, it should also impact cell-cycle progression and cell proliferation.

A significant change was observed in cell distribution during the cell cycle in CPEB1-depleted cells, indicating ~~a~~ delayed exit from the G2/M phase (Figure 5D). This effect was enhanced when CPEB1 and miR-15b were depleted simultaneously (Figure 5D). This result is consistent with the observation that depletion of WEE1 accelerates the G2/M phase in *Xenopus*. Here, depletion of CPEB1 and miR-15b increased WEE1 expression and resulted in delayed exit from G2/M.

Cell growth was also affected by CPEB1 depletion (Figure 5E), as documented by the decreased number of cells; however, inhibiting miR-15b had little effect on cell counts. Taken together, these data show that WEE1 is regulated at the translational level by CPEB1 and miR-15b in a coordinated and cell-cycle-dependent manner.

Discussion

We have analyzed the regulation of WEE1, an oncogenic cell-cycle regulator, by the RNA binding complexes CPEB1 and miR-15b/RISC. We show that miR-15b/RISC and CPEB1 have a coordinated impact on WEE1 translation. There are few examples of coordination

between RISC and other RNA binding proteins. Under stress conditions, the inactivation, storage, and reactivation of CAT-1 mRNA depend on the binding of both miR-122 and HuR, an AU-rich-element-binding protein, to the 3'UTR of CAT-1 mRNA [28]. Cooperation between miR-16 (a member of the miR-15/16 family of miRNPs) and an ARE-binding protein called TTP (tristetraprolin) was shown to occur through the association of TTP with AGO/eIF2C family members. MiR-16 assists TTP in targeting ARE, which appears to be an essential step in ARE-mediated mRNA degradation [29]. Our data not only constitute an additional example of such a coordinated action, but also demonstrate that such a coordinated action can be cell-cycle-dependent, the two sites being inhibitors during G1 and S and activators during G2/M. In G1/S, the two regulators are at least in part colocalized in cytoplasmic granules (Figure 2D), which may be silencing granules (since they include DDX6/p54, Figure S2). These granules disappear at G2/M [30] and Figure S4A. This could at least in part account for decreased inhibition by CPEB1 and miR-15b at G2/M. However, we observed that at G2/M the two sites not only lost their inhibitory activity, but also had a tendency to activate *WEE1* mRNA translation, since their deletion decreased both the activity of a reporter construct (Figure 4B and Figure S4C) and the ribosome loading of the reporter mRNAs, as monitored by their distribution in heavy polysomal fractions (Figure 4D). Moreover, depletion of CPEB1 and miR-15b affected the expression of the endogenous protein in an opposite manner in G1/S or G2/M cells (Figure 5A, B). CPEB1 is known to act as a cell-cycle-dependent activator of translation by elongating mRNA poly(A) tails during *Xenopus* oocyte maturation and during the transition to M phase in *Xenopus* embryo [9, 31]. But such a cell-cycle-dependent switch is much less expected for the RISC complex, and translational activation by RISC complexes

is a rather controversial issue. There are, however, examples of such switches in the RISC complex in the literature. It has been shown that upon cell-cycle arrest, the ARE (AU-rich element) in tumor necrosis factor-α (TNFα) mRNA is transformed into a translation activation signal, recruiting AGO and fragile X mental retardation-related protein 1 (FXR1), factors associated with miRNPs. Human miRNA miR-369-3 directs the association of these proteins with the AREs, leading to translation activation (Vasudevan et al., 2008). Moreover, two well-studied miRNAs – *let-7* and the synthetic miRNA miRcxcr4 – likewise induced translational up-regulation of target mRNAs upon cell-cycle arrest, yet they repressed translation in proliferating cells. It has been proposed that translation regulation by miRNPs oscillates between repression and activation during the cell cycle [32]. It is quite possible that switches of activity by the RISC complex depend on the switch in activity of other 3'UTR-binding proteins, such as CPEB1. Indeed, here, in the absence of CPE, miR-15b did not activate WEE1 3'UTR in a reporter construct.

CPEB1 activity is regulated by phosphorylation during the *Xenopus* embryo cell cycle [10]. It is likely that similar phosphorylation is involved in HeLa cells. Phosphorylation, possibly by the same enzyme, might also be responsible for the modification of miR-15b /RISC activity. In *Xenopus* embryo, CPEB1 phosphorylation results in the modification of the composition of the CPEB1 complex, which recruits a polymerase rather than a deadenylase. Thus, we speculate that at G2/M, CPEB1 and miR-15b/RISC recruit activating proteins rather than inhibitors, possibly increasing the size of the poly(A) tail of WEE1 mRNA. Most interestingly, whereas the inhibiting activities of the two complexes were acting independently of each other, activation required the integrity of each of the two sites, implying that the two complexes cooperate for this action. This was further supported

by the observation that independently mutating each site also reduced the association of WEE1 mRNAs with the heavy polysomal fraction at G2/M, indicative of reduced translation. Thus, it appears that whereas each of the complexes can independently recruit translation inhibitors, they need to cooperate to recruit translation activators. The mechanism of this cooperation is elusive. The two complexes do not appear to interact directly in unsynchronized cells, which are mostly in G1/S, since most of the interaction is sensitive to RNase (Figure 3A). It is quite possible however, that the two complexes do interact in G2/M cells, which would account for the residual binding observed after RNase treatment (Figure 3A), since a small fraction of cells are in G2/M in the unsynchronized population. We will try to verify it in our future work.

In summary, here we demonstrate a cell cycle-dependent, coordinated action of a miRNA (miR-15b) and an RNA-binding protein (CPEB1) to inhibit *WEE1* mRNA translation in G1/S and activate it in G2/M, providing an example of coordination between a miRNA/RISC complex and another RNA binding protein, as well as, even more importantly, of a cell cycle-dependent switch from inhibitor to activator of the two complexes.

Materials and methods

Cell lines and constructs

HeLa and HeLa S3 cells were maintained in DMEM media supplemented with 10% FCS, 100 mg/ml streptomycin, and 100 U/ml penicillin (Gibco®) cultured at 37ºC and 5% CO_2. The *WEE1* 3'UTR sequence (nt 2274-2524, [GeneBank: NM 001143976.1]) was cloned into XbaI/NotI sites in the pRL-TK vector (Promega). An miR-15b site substitution mutation was introduced by replacing the 2316-2325 sequence in the *WEE1* 3'UTR by

accacacgcga. CPE site mutation was accomplished by replacing ttttttaatt (2494-2504) with gggggggccgg in the *WEE1* 3'UTR. Double miR-15b and CPE mutations were done by simultaneous replacement of both sites.

CPEB1 transcript variant 1 [GeneBank: NM_030594.3] was cloned into XhoI/EcoRI sites of the pmCherry-N1 expression vector.

In transfection assays, pmCherry-N1 (632523, Clontech) was used as control.

MiRNA precursor molecules (Applied Biosystems) used were: miR-15b (PM17100);

MiRNA inhibitors (Applied Biosystems) used were: miR-15b (AM17000) and Negative Control #1 (PM17110).

SiRNAs used were: siCPEB1 (5' taggaggtgttccttgggata 3', 5'cagccgaaggatgcgctgcaa 3'), and AllStars - negative control siRNA (Qiagen).

Transfection assays and luciferase measurements

All transfections were performed in triplicate in 96-well plates with 200ng (or 100ng + 100ng) DNA and lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Pre- and anti-miRNAs were used at 50nM and siRNAs at 10nM. Cells were lysed after 20h of transfection using Passive Lysis Buffer (Promega), then Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System Kit (Promega). Results are expressed as *Renilla* luciferase values normalized to Firefly, and statistical analyses performed using Student's *t* test.

Quantitative PCR

Total RNA purification was performed using Trizol (Invitrogen) according to the manufacturer's instructions. 500ng or 250ng total RNA was used for *CPEB1,WEE1 mRNA*

quantification, using QuantiTect SYBR-green RT-PCR kits (Qiagen) and a LightCycler Real-Time PCR System (Roche).

For miRNA analysis, 25ng of total RNA was reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). QPCR was carried out in triplicate using the TaqMan Universal PCR Master Mix (Applied Biosystems).

Primers used were: *WEE1* forward (5' acctcggataccacaagtgctt 3')
reverse (5' ggtcttctctctggatctggatga 3'), WEE1 3'UTR forward (5' ttggatgttacaccagcctt 3'), WEE1 3'UTR reverse (5' gatatacaagtctaattcaca 3'); CPEB1 forward
(5' tcccagatgcaaatgacttgtgcc 3'), *CPEB1* reverse (5' aacttgtccaccaagtcagaccca 3');
(5' gacgatgcgctagatgtcaa 3'); *GAPDH* forward (5' ggacctgacctgccgtctagaa 3'), *GAPDH* reverse (5' ggtgtcgctgttgaagtcagag 3'); Cyclophiline A forward (5'-GTCAACCCCACCGTGTTCTT-3'); Cyclophilin A reverse (5'-CTGCTGTCTTTGGGACCTTGT-3')

Immunoprecipitation assay

Four 15-cm dishes of HeLa S3 cells were transfected with Myc-FLAG-CPEB1, HA-FLAG-CPEB1 (or CPEB1 mutants), or HA-FLAG-AGO2 expression plasmids (OriGene). After 48h of incubation, cells were collected, lysed in 150ml/plate lysis buffer consisting of 100mM KCl, 5mM $MgCl_2$, 20mM Hepes-KOH (pH 7.8), 2mM DTT, 0.25% NP-40, and protease inhibitor cocktail (Roche) for 30 min on ice.

Lysates were incubated with 100ml anti-FLAG beads (SIGMA), washed in lysis buffer, and incubated overnight at 4°C with rotation. The beads were washed 5 times in 0.5ml lysis

buffer. Then CPEB1 complexes were eluted in 120ml of lysis buffer with 100mg/ml FLAG peptide at 4°C for 2h with shaking. FLAG peptide elution were analyzed by Western blot.

SDS-PAGE and Western Blot

Protein extracts were prepared in 100mM KCl, 5mM $MgCl_2$, 20mM Hepes-KOH (pH 7.8), 2mM DTT, 0.25% NP-40, and protease inhibitor cocktail (Roche).

20mg of protein was loaded onto NuPAGE 4-12% Bis-Tris Pre-Cast Gels and resolved using the NuPAGE System (Invitrogen), followed by Western blotting, using standard techniques. Primary antibodies and dilutions used were: CPEB1 1:1000 (NB100-1437, Novus Biological), b-actin 1:10000 (A5441, Sigma) and α-Tubulin 1:5000 (F2168, Sigma), WEE1 (sc-5285, Santa Cruz), Cyclin B1 (sc-752, Santa Cruz), Myc-tag (sc-40, Sigma), AGO1 (SAB4200084, SIGMA), AGO2 (SAB4200085, SIGMA). Secondary antibodies and dilutions used were anti-rabbit-HRP 1:25000 (A0545, Sigma), anti-mouse 1:25000 (A2304, Sigma), and anti-rat 1:25000 (A9037, SIGMA).

Protein levels determined by Western blot were quantified using BioImage software.

Ribosomal profile

15-cm plates of HeLa S3 cells were transfected with pRL-TK Vector containing a fragment of the *WEE1* 3'UTR (wt, or else mutated at the miR-15b and/or CPE sites) attached to the luciferase coding sequence. After 24h, cells were lysed in 500ml buffer containing 20mM Tris-HCl (pH 8.5), 1.5mM $MgCl_2$, 140mM KCl, 0.5mM DTT, 0.5% NP-40, 1000U of RNAsin (Promega) per ml, and 0.1mM cycloheximide. The lysate was centrifuged at 10,000*g* for 10 min, and supernatants were applied to linear 20-47% sucrose gradients in

buffer containing 20mM Tris-HCl (pH 8.0), 140mM KCl, 5mM $MgCl_2$. Centrifugation was carried out at 40,000 rpm for 135 min in a Beckman SW41 rotor. 1 ml fractions were collected using a Teledyne ISCO Model 160 system with concomitant measurement of the absorbance at 260nm followed by RNA precipitation by 1ml of isopropanol and Q-PCR analysis with primers for Luciferase (forward: 5' atttaggtgacactatacatgacttcgaaagtttatg 3' and reverse: 5' taatacgactcactatacgcaatatcttcttcttcaatatc 3'), with GAPDH (forward: 5' ggacctgacctgccgtctagaa 3'and reverse: 5' ggtgtcgctgttgaagtcagag 3') as control.
Cell synchronization experiments were done in a similar way, but cells were transfected with plasmid after release from a double thymidine block. Cells were collected 29h after transfection, lysed, and separated on a sucrose gradient. RNA were extracted and subjected to Q-PCR analysis.

Cell synchronization and FACS analysis

HeLa S3 cells (25-30% confluence) were incubated with 2mM thymidine for 18 hours. Cells were then washed in PBS and incubated in DMEM (10% FBS) for 9 hours to release them from the first thymidine block. 2mM thymidine was then added to DMEM for 17 hours (second thymidine block). After removal of thymidine by washing in PBS and adding fresh DMEM (10% FBS), cells were transfected with luciferase reporter constructs and 24 hours later some cells were used for the luciferase reporter assay and some were fixed in 70% EtOH at 4°C overnight. The next day, fixed cells were subjected to propidium iodide (PI) labeling. Cells were washed with PBS twice, resuspended in 50mL PBS containing a final concentration of 1mg/mL RNAse (DNAse-free, Qiagen), incubated at 4°C for 5 min, then added to 45 mL PBS containing 50mg/mL PI (final concentration), incubated at 4°C for 30 min in the dark and subjected to FACS analysis by FACScan (Becton Dickinson).

In situ hybridization

Antisense and control (sense) probes for in situ hybridization were prepared by incorporating fluorescein-12-UTP (11 427 857 910 Roche) into the luciferase mRNAs respectively transcribed in vitro from T7 and SP6 promoters, using the mMessage mMachine kit (Ambion); the coding sequence was obtained by PCR amplification of a DNA fragment using as primers:

forward, 5' atttaggtgacactatacatgacttcgaaagtttatg 3';

reverse, 5' taatacgactcactatacgcaatatcttcttcttcaatatc 3'.

HeLa cells were fixed for 15 min in 3.7 % PFA in PBS at RT, washed in PBT (0.1% Tween-20 in PBS) and stored in 70% EtOH at 4°C overnight. After rehydration in PBT, cells were treated for 3 min with 1mg/mL Proteinase K in PBT, washed in 2mg/mL glycine in PBT and post-fixed for 10 min in 3.7% PFA in PBT. Cells were initially incubated for 1 hour in hybridization mix (HM; 50% formamide, 5xSSC, 1x Denhardt's solution, 200 mg/mL yeast tRNA, 500mg/mL salmon sperm DNA, 2% blocking reagent (Roche)) at 37°C, and then hybridized overnight with 40nM probe at 37° C. Post-hybridization washes were carried out for 3 x 15 min in 30% formamide/2x SSC at 50° C, 1 x 15 min in 2x SSC at RT, and 1 x 15 min in 0.2x SSC at RT. Cells were rinsed three times with PBS then rinsed once for 2-5 min with Hoechst 33258 (the 16mM stock solution was diluted 1:8000 final concentration 2mM), mounted on glass slides with Dako mounting medium, and analyzed by fluorescence microscopy.

Co-transfection of labeled molecules and confocal microscopy

FITC-labeled *WEE1* 3'UTR mRNAs were prepared by incorporation of fluorescein-12-UTP (Roche) into the in-vitro transcript obtained from the T7 promoter using the mMessage mMachine kit (Ambion); the coding sequence was obtained by PCR amplification of a DNA fragment from Rluc-*WEE1* 3'UTR constructs using as primers:

forward, 5' taatacgactcactatacgttcgttgagcgagttctc 3';

reverse, 5' atttaggtgacactatacactgcattctagttgtggt 3'.

Mir-15b precursor (Applied Biosystems) was labeled with Cy5 (Label IT siRNA Tracker Cy5 Kit, Mirus) according to the company protocol.

FITC-labeled *WEE1* 3'UTR (wt, CPE and/or miR-15b site mutations), Cy5-labeled miR-15b precursor and/or CPEB1-Cherry DNA were cotransfected into HeLa cells with a Lipofectamin 2000 Kit (Invitrogen) according to the company protocol. Cells were fixed 48 hours later in 4% paraformaldehyde, labeled, and visualized by confocal microscopy.

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Figure legends

Figure 1. Regulation of WEE1 in HeLa cells depends on CPE and miR-15b binding sites. (**A**) Cell cycle distribution (by FACS analysis) of HeLa cells at indicated times after release from a double thymidine block. (**B**) Western blot analyses of WEE1 expression in HeLa cells after release from a double thymidine block; actin is used as a loading control. (**C**) QPCR analyses of WEE1 mRNA, in the same cells; Cyclo A was used to standardize the data. (**D**) Schematic representation of R-luc *WEE1* 3'UTR fusions (WEE1 3'UTR was introduced into the 3'UTR of Renilla luciferase reporters). (**E**) Luciferase expression of the reporter constructs described in D following transfection into HeLa cells; *Renilla* enzymatic activity (LucR) was normalized to Firefly (control, LucF).

Statistics: Student's *t* test; *: t = 0.05.

Figure 2. MiR-15b and CPEB1 control WEE1 expression. (**A**) Depletion of CPEB1 by siRNAs (Western blot). (**B**) Depletion of miR-15b by an antisense LNA (QPCR); (**C**) Influence of CPEB1 and/or miR-15b depletion on *WEE1* 3'UTR reporter luciferase activity transfected into HeLa cells along with inhibitors as indicated. Controls were miRNA (Applied Biosystems) and AllStars (Qiagen). Numbers indicate the ratio LucR/LucF for each sample. (**D**) Localization of WEE1 mRNA, CPEB1, and miR-15b in HeLa cells. HeLa cells were transfected with Cy5-labeled miR-15b, CPEB1-Cherry, FITC-labeled *WEE1* 3'UTR (wt or mutant as indicated). Arrows point to granules with co-localized *WEE1* mRNA, CPEB1, and/or miR-15b. (**E**) CPEB1 and miR-15b control WEE1 protein expression. For gain-of-function assays, HeLa cells were transfected with a construct expressing CPEB1-Cherry (or Cherry as a control), or with miR-15b precursor (or a control miRNA); for loss-of-function assays, cells were transfected with the inhibitors described in

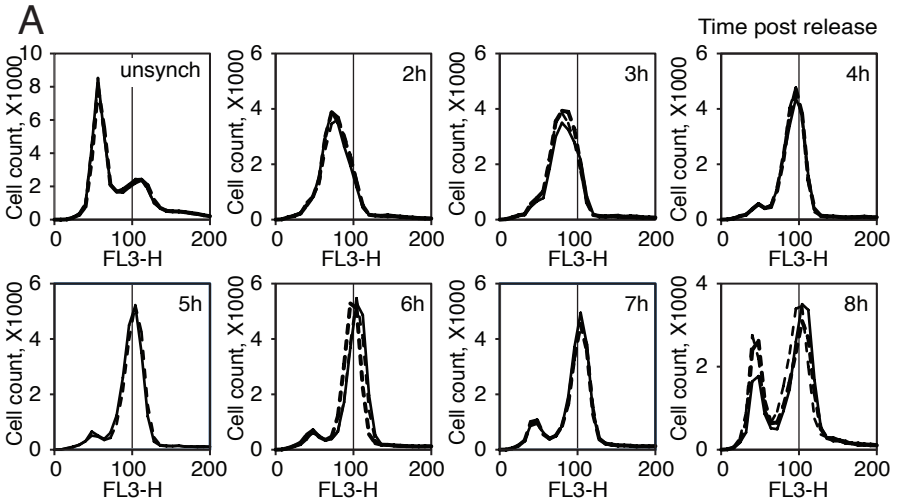
A and B; WEE1 expression was monitored 48h later by Western blot. (**F**) Overexpression of miR-15b monitored by QPCR. (**G**) Overexpression of CPEB1 monitored by Western blot.

Figure 3. Interaction between CPEB1 and AGO2 is RNAse and DDX6 dependent. (**A**) Co-immunoprecipitation of endogenous AGO2 with CPEB1-Myc-FLAG; cells were transfected with a tagged CPEB1 (CPEB1-Myc-FLAG) expression vector or an empty vehicle vector as a control (vector); cell extracts were immunoprecipitated using anti-FLAG antibodies, following RNase treatment when indicated, and monitored by Western blot; inp: input; IP: immunoprecipitates. (**B**) Cells were transfected with a tagged AGO2 (AGO2-FLAG-HA) expression vector or an empty vehicle vector as a control (vector); cell extracts were immunoprecipitated using anti-FLAG antibodies, following RNase treatment when indicated. (**C**) DDX6 is required for AGO2/CPEB1 interaction. Cells treated for 48h with an siRNA to DDX6 (or a control siRNA) were transfected and treated as in A. (**D**) Cells were transfected with CPEB1-Myc-FLAG and AGO2-FLAG-HA expression vectors, along with wild type (wt) or transdominant DDX6 helicase dead mutant (mut) expression vectors; extracts were immunoprecipitated with anti-HA antibodies and analyzed as in A. (**E**) Cells were transfected with wild-type DDX6 (wt) or transdominant DDX6 mutant (mut) together with CPEB1-Myc-FLAG or AGO2-FLAG-HA expression vectors; extracts were immunoprecipitated with anti-FLAG antibodies and analyzed as in A and B.

Figure 4. CPEB1 and miR-15b inhibitors switch to activators at G2/M. (**A**) Cell-cycle analysis (by FACS) of HeLa cells after release from a double thymidine block unsync, -

unsynchronized cells. (**B**) HeLa cells analyzed in A were transfected after they were released from thymidine block with *WEE1* 3'UTR reporter constructs (described in Figure 1D) and 20h later, cell extracts were submitted to luciferase assays. *Renilla* luciferase activity was normalized to Firefly activity; the position of the majority of the cells in the cell cycle in the samples is indicated above the bars; Statistics: Student's *t* test; *: t = 0.05. (**C**) WEE1 expression along the cell cycle as monitored by Western blot; cyclin B1 is shown to document the position of the cells in the cell cycle. (**D**) Distribution of WEE1 3'UTR reporter mRNAs in polysomal gradients at the G0/G1 or G2/M phase of the cell cycle. Fractions 0-3 correspond to monosomal fractions, 4-7 to low polysomal fractions, and 8-12 to heavy polysomal fractions.

Figure 5. Depletion of CPEB1 or miR-15b affects both the cell-cycle-dependent accumulation of WEE1 and cell-cycle progression. (**A**) Cells were transfected with a siRNA to CPEB1, an antisense to miR-15b, or control molecules as indicated and submitted to a double thymidine block as in Figure 4; WEE1 or Cyclin B1 expression was monitored by Western blot. (**B**) Quantification of the Western blots shown in (A) after standardization to actin. (**C**) WEE1 mRNA expression in the same cells, monitored by Q-RT-PCR. (**D**) Cell-cycle distribution (analyzed by FACS) of HeLa cells after depletion of CPEB1 and/or miR-15b. (**E**) HeLa cell-count after CPEB1 and/or miR-15b depletion.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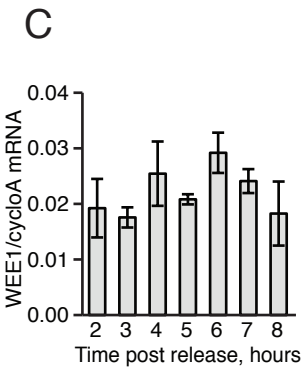
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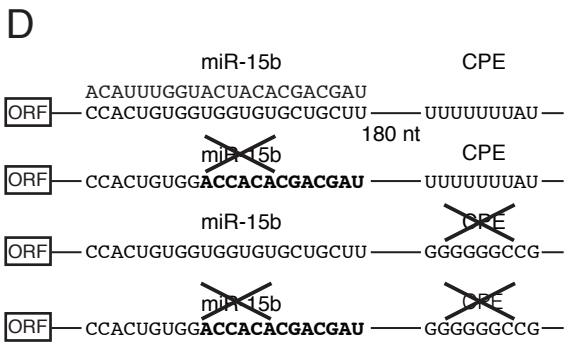
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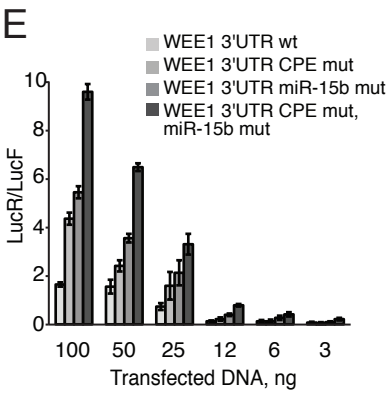
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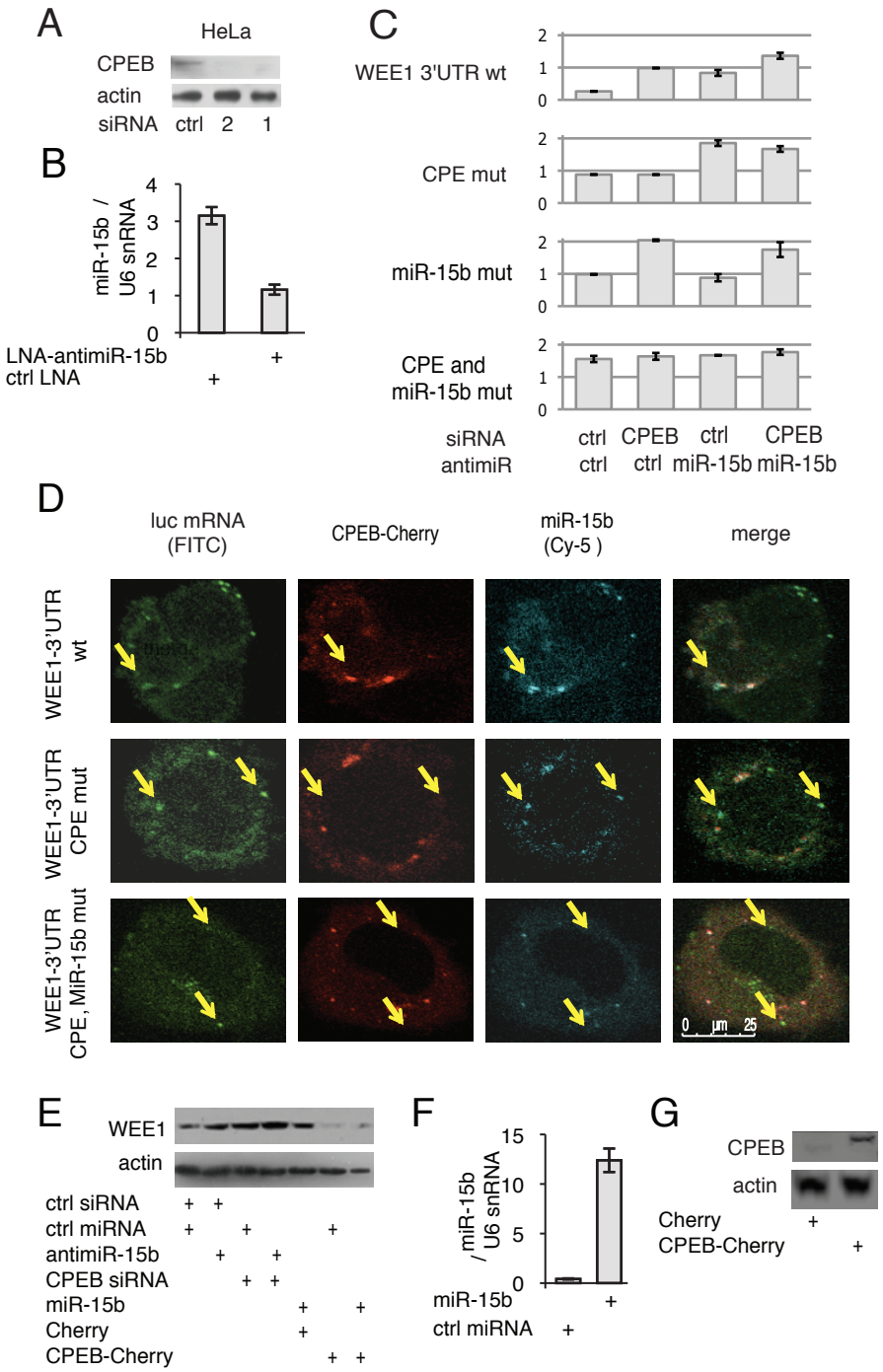
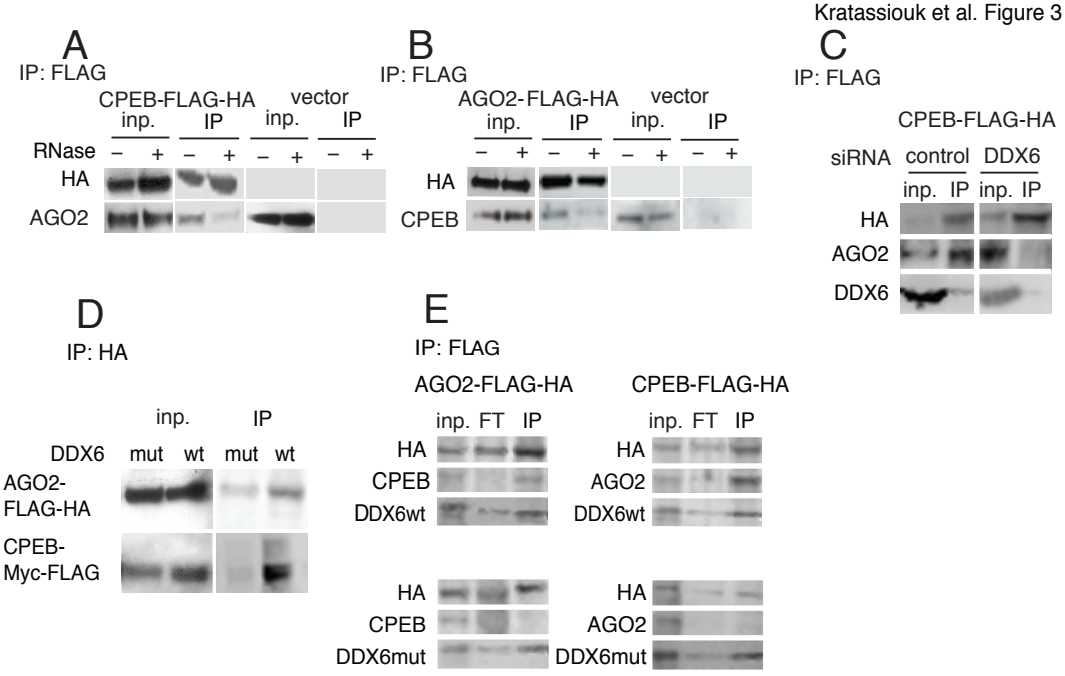
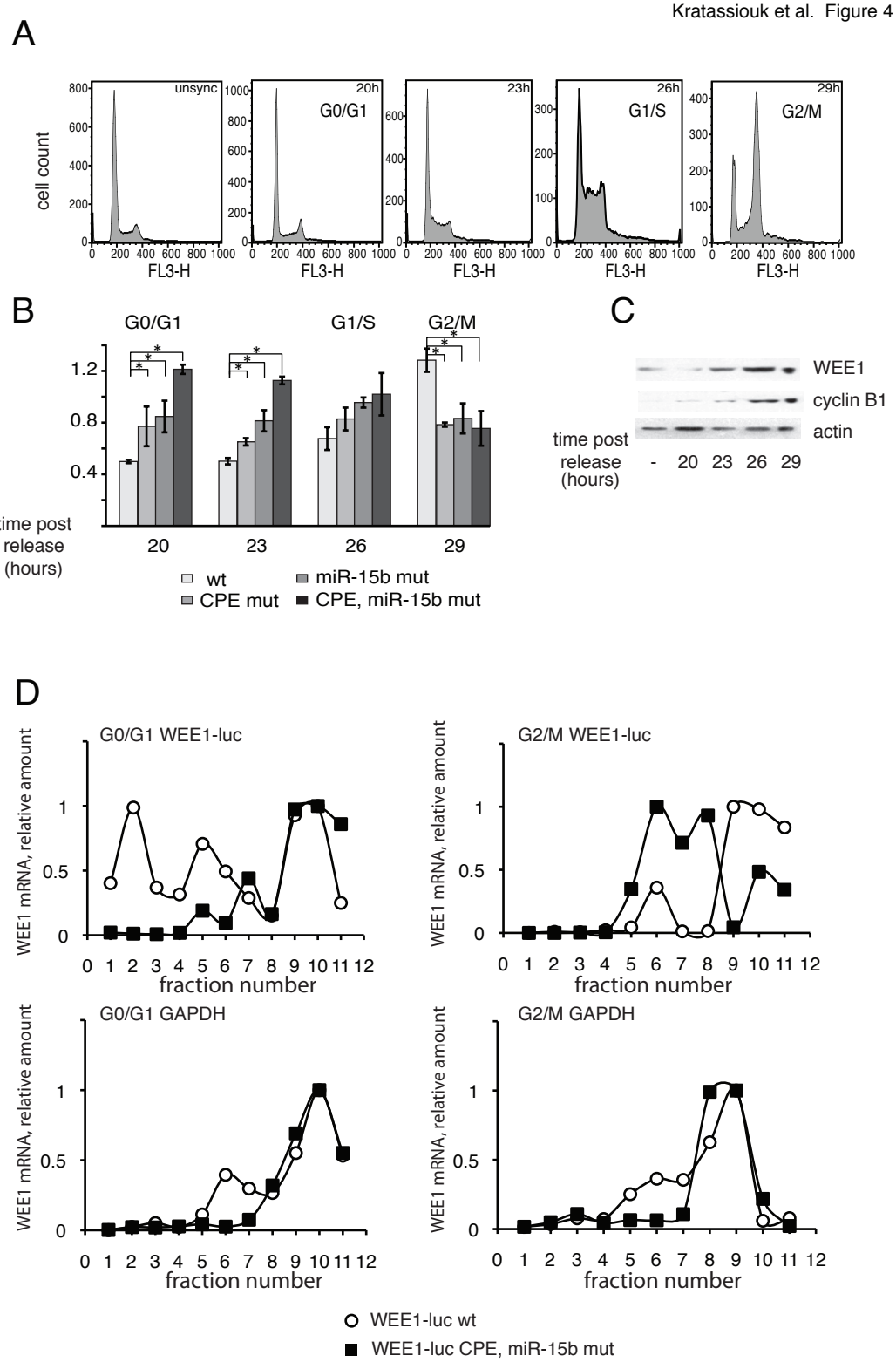
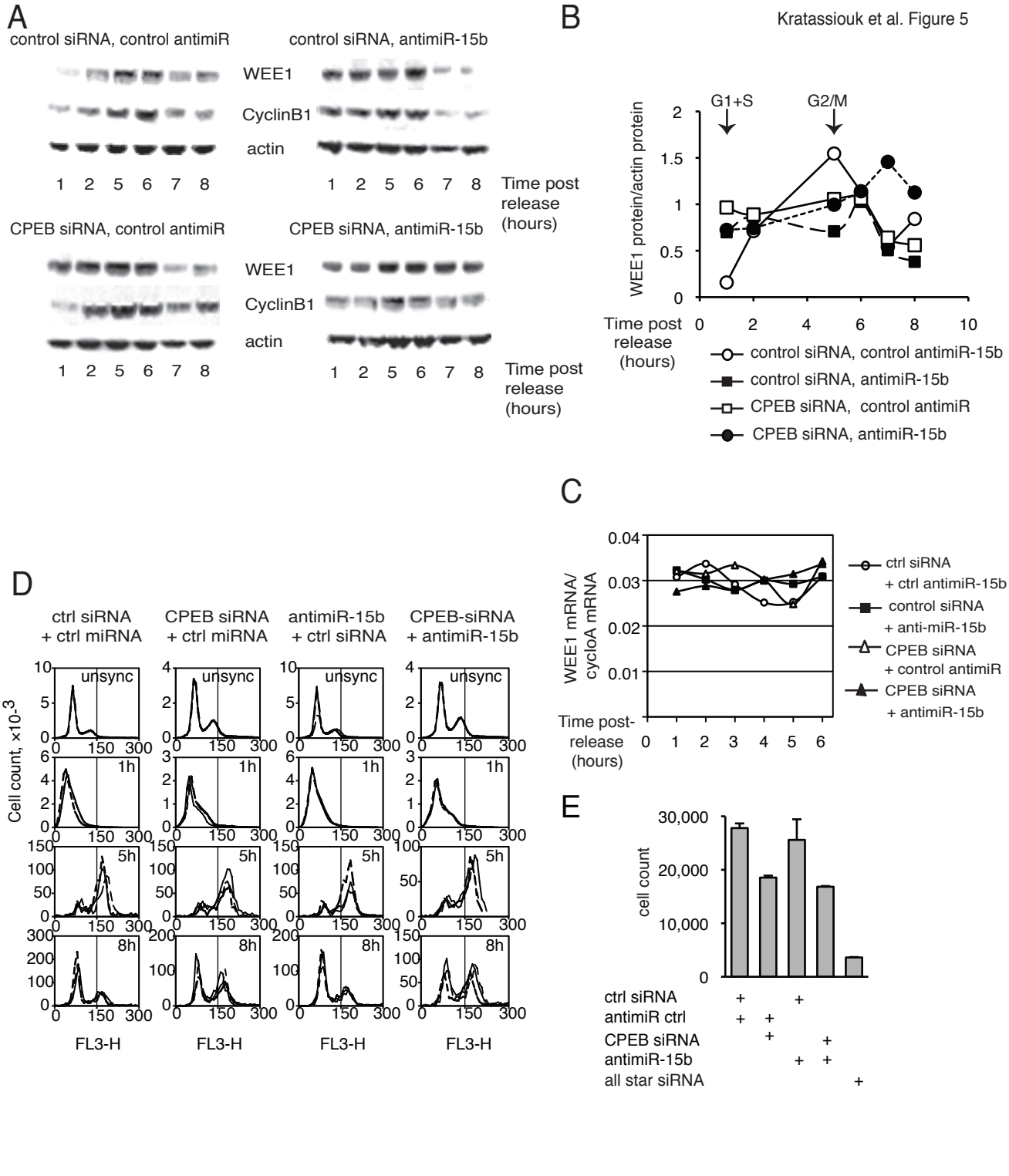
LucR/LucF
Transfected DNA, ng


A
HeLa
CPEB
actin
siRNA ctrl 2 1
B
miR-15b / U6 snRNA
LNA-antimiR-15b
ctrl LNA
C
WEE1 3'UTR wt
CPE mut
miR-15b mut
CPE and miR-15b mut
siRNA ctrl ctrl CPEB ctrl CPEB
antimiR ctrl miR-15b miR-15b
D
luc mRNA (FITC)
CPEB-Cherry
miR-15b (Cy-5)
merge
WEE1-3'UTR wt
WEE1-3'UTR CPE mut
WEE1-3'UTR CPE, MiR-15b mut
E
WEE1
actin
ctrl siRNA
ctrl miRNA
antimiR-15b
CPEB siRNA
miR-15b
Cherry
CPEB-Cherry
F
miR-15b / U6 snRNA
miR-15b
ctrl miRNA
G
CPEB
actin
Cherry
CPEB-Cherry

Kratassiouk et al. Figure 3




A
unsync
20h
G0/G1
23h
26h
G1/S
29h
G2/M
cell count
FL3-H
B
G0/G1
G1/S
G2/M
time post release (hours)
20
23
26
29
wt
miR-15b mut
CPE mut
CPE, miR-15b mut
C
WEE1
cyclin B1
actin
time post release (hours)
- 20 23 26 29
D
G0/G1 WEE1-luc
G2/M WEE1-luc
G0/G1 GAPDH
G2/M GAPDH
WEE1 mRNA, relative amount
fraction number
WEE1-luc wt
WEE1-luc CPE, miR-15b mut


A
control siRNA, control antimiR
control siRNA, antimiR-15b
WEE1
CyclinB1
actin
1 2 5 6 7 8
Time post release (hours)
CPEB siRNA, control antimiR
CPEB siRNA, antimiR-15b
B
G1+S
G2/M
WEE1 protein/actin protein
Time post release (hours)
control siRNA, control antimiR-15b
control siRNA, antimiR-15b
CPEB siRNA, control antimiR
CPEB siRNA, antimiR-15b
C
WEE1 mRNA/ cycloA mRNA
Time post-release (hours)
ctrl siRNA + ctrl antimiR-15b
control siRNA + anti-miR-15b
CPEB siRNA + control antimiR
CPEB siRNA + antimiR-15b
D
ctrl siRNA + ctrl miRNA
CPEB siRNA + ctrl miRNA
antimiR-15b + ctrl siRNA
CPEB-siRNA + antimiR-15b
unsync
1h
5h
8h
Cell count, x10-3
FL3-H
E
cell count
ctrl siRNA
antimiR ctrl
CPEB siRNA
antimiR-15b
all star siRNA

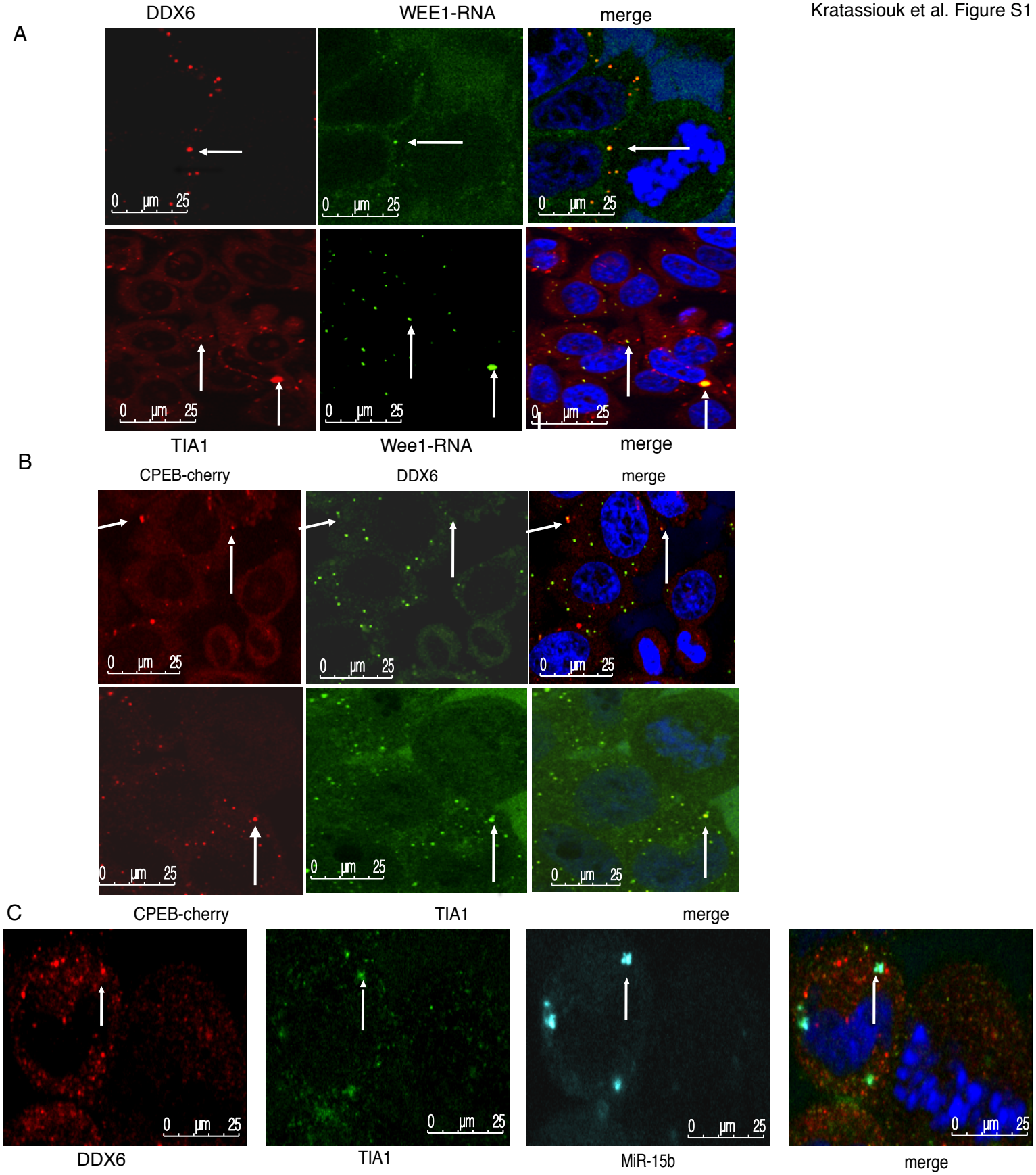


Figure S1. FITC-labeled WEE1 RNA, Cy5 miR-15b, and CPEB1-Cherry are localized in P-bodies and stress granules. (**A**) Cells transfected with FITC-labeled *WEE1* RNA fixed 24 h later and immunostained with DDX6 (P-body marker) and TIA (stress granule marker) antibody and subjected to confocal microscopy. (**B**) Cells transfected with CPEB1-Cherry were immunostained with DDX6 and TIA antibody and subjected to confocal microscopy. (**C**) Cells transfected with Cy5-miR-15b precursor were fixed 24 h later and immunostained with DDX6 (P-body marker) and TIA (stress-granule marker) and subjected to confocal microscopy

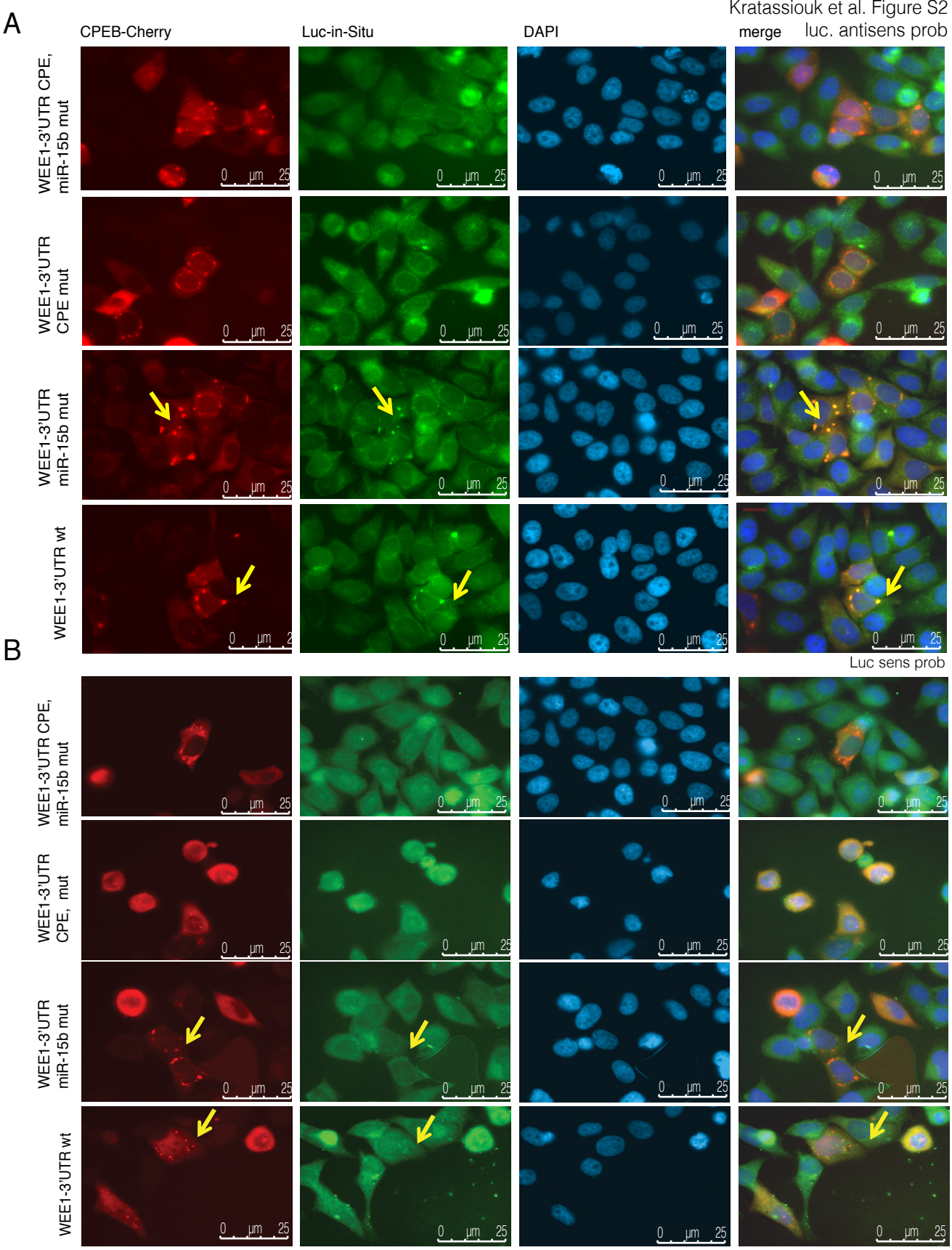


Figure S2. WEE1 3'UTR-dependent distribution of R-Luc mRNA to silencing granules in HeLa cells. (A) Cells were transfected with CPEB1-Cherry and R-Luc WEE1 3'UTR (wt or mutant as indicated) DNA constructs and, after 48h of incubation, fixed and used for in-situ hybridization with R-Luc anti-sense probe and visualized by fluorescence microscopy (Arrows point to CPEB1-granules that co-localized with WEE1 3'UTR). (B) Cells were transfected with CPEB1-Cherry and R-Luc WEE1 3'UTR constructs and treated as in (A) (R-Luc sense probe used for control). Arrows point to CPEB1-granules.

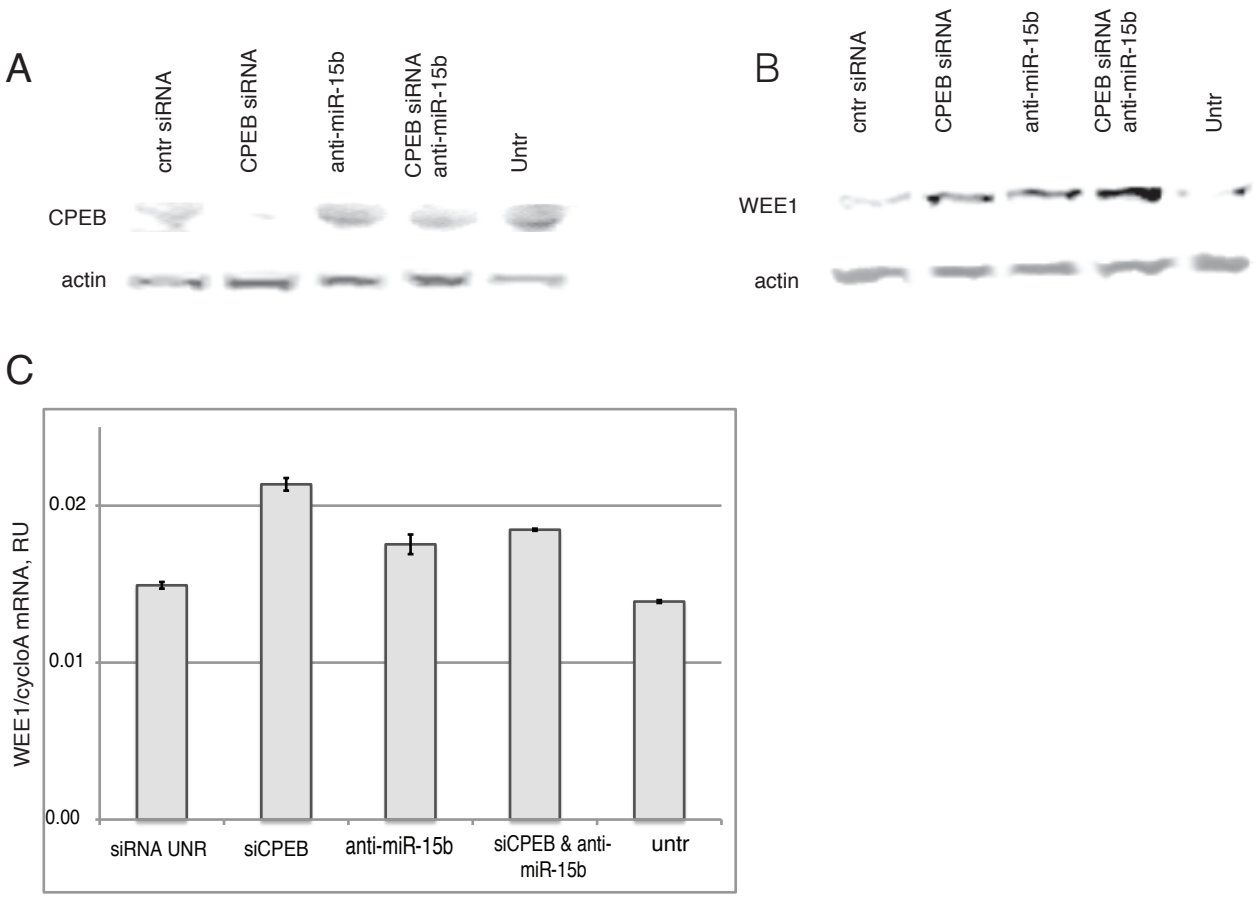


Figure S3. CPEB1 siRNA knock-down and/or miR-15b LNA down-regulation stimulate WEE1 expression**.**

(**A**) Depletion of CPEB1 by siRNAs. Western blot analyses of CPEB expression performed 48 h after HeLa cells were transfected with CPEB-siRNA and/or miR-15b LNA antisense probe. (**B**) CPEB1 and miR-15b control WEE1 protein expression. Western blot analyses of WEE1 expression performed 48 h after HeLa cells were transfected with CPEB1-siRNA and/or miR-15b LNA antisense probe. (**C**) CPEB1 mRNA expression is not under CPEB1 and miR-15b control. Q-PCR analyses of WEE1 mRNA accumulation in HeLa cells, performed 48 h after cells were transfected with CPEB1-siRNA and/or miR-15b LNA antisense probe.

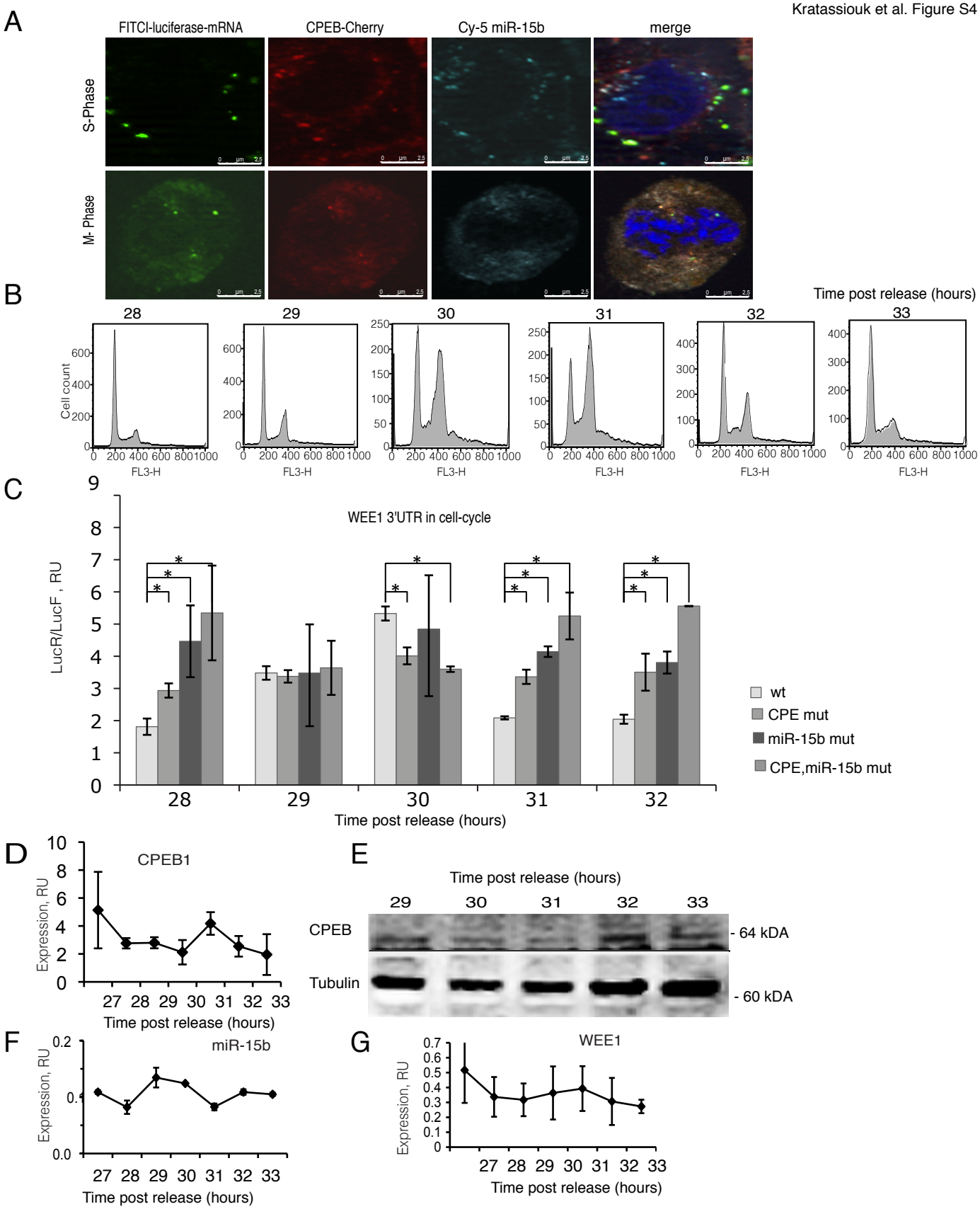


Figure S4. MiR-15b, WEE1 and CPEB1 mRNA, protein expression and localization during cell cycle in HeLa. (**A**) HeLa cells were synchronized by double thymidine block and transfected, after release, with FITC-labeled wt WEE1 3'UTR, CPEB1-Cherry, and Cy5-labeled miR-15b, incubated for 29 h, fixed, and analyzed by confocal microscopy (Leica TCS SP5 II). (**B**) FACS analyses of HeLa cells performed every hour-started 28h after release from double thymidine block. (**C**) HeLa cells were transfected with R-luc wt WEE1 3'UTR or CPE and/or miR-15b mutant reporter constructs after release from a double thymidine block. Luciferase reporter assays were carried out every hour started 28h after transfection (**D, F**). CPEB1 mRNA and protein expression during cell cycle. QPCR and Western blot analyses of CPEB1 mRNA and protein performed at the same time points as in C; (**E**) QPCR analyses of the expression levels of miR-15b performed at the same time points as in C.

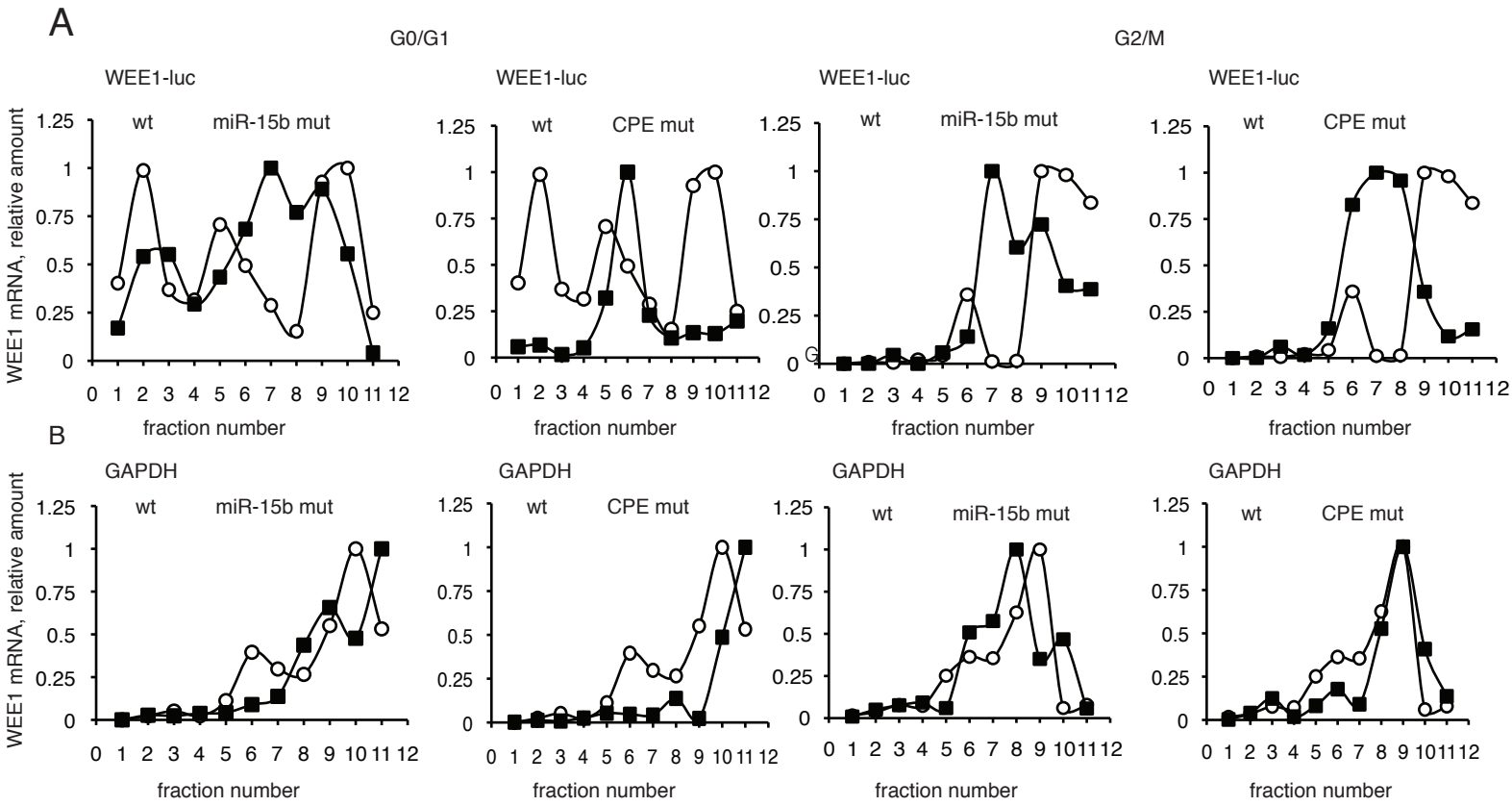


Figure S5. R-luc-WEE1 3'UTR mRNA presentation in polysomal gradient
(**A**) Distribution of R-luc-WEE1 3'UTR mRNA with CPE or miR-15b binding site mutant in polysomal gradient during G0/G1 and G2/M phase of cell cycle. (**B**) Distribution of GAPDH mRNA (internal control) across the polysomal gradient during G0/G1 and G2/M phase of cell cycle.

Figure S6. Differential expression of WEE1 along the cell cycle is dependent on DDX6. HeLa cells were transfected with control SiRNA and DDX6 SiRNA, Cells were synchronized by double thymidin block. Western blot analyses of WEE1 and cyclin B1 expression were performed at each hour after release from the double thymidine block!

Conclusions

1) IMP3 regulates the expression of cyclin D1 and D3 in a significant manner in vivo. Inhibition of IMP3 did not significantly change the mRNA levels of cyclin D1 and D3. The stability of cyclin D1 and D3 mRNAs does not depend on the presence of IMP3.
2) In the absence of IMP3 cyclin D1 and D3 mRNAs are exported to the cytoplasm and seems to have preferentially perinuclear localization.
3) After IMP3 KD cyclin D1 and D3 mRNAs are associated with polyribosomes, but not translated.
4) In the cytoplasm of RMS cells IMP interact with multiple RBPs, suggesting an existing of a RNP complex, that positively regulate cyclin D1 and D3 translation.
5) We found that the decrease of the cyclins D1, D3 protein levels after IMP-3 or its protein partners (NF90 and PTBP1) KD can be reversed by KD of two key components of RISC complex: GW182 and Ago2.
6) We identified miR let-7, as well as miR 15 and miR 193b (data not shown) for cyclin D1 and miR let-7 for cyclin D3, that can be involved in miRISC-dependent translational repression of these cyclins. The efficiency of miRNA-dependent translational repression can be regulated by IMP-3 and RISC complex components.
7) We determined functional regions within cyclin D1 and D3 3'UTRs, where IMP3 and miRISC complexes compete for cyclin regulation.

Taken together, the results of this work show, that IMP3 and its protein partners form a complex on 3'UTRs of the cyclins D1 and D3 and protect them from miRISC-dependent translational repression.

DISCUSSION

IMP3 regulates cyclin D1 and D3 expression at post-transcriptional level.

RNA-binding proteins in cells associate with their target mRNAs, resulting in ribonucleoprotein (RNP) complexes formation. The composition of such complexes determines the cellular fate of mRNAs: localization, translation and decay. IMPs form stable protein-RNA associations that can prevent premature decay and promiscuous translation of specific target transcripts, as it has been shown for CD44, MYC, PTEN or BTRC [Lemm I and Ross J, 2002; Kobel M et al., 2007; Stohr N et al., 2012 ; Vikesaa J et al., 2006 ; Noubissi FK et al., 2006]. By regulating these and other mRNA targets, IMP3 was reported to promote tumor progression and metastasis in a number of malignancies.

We have previously shown that IMP3 regulates the expression of cyclin D1 and D3 in a significant manner in vivo [Rivera Vargas, Boudoukha et al. 2014]. Cyclins are short-lived proteins with two- to four-fold shorter half-lives than their corresponding mRNAs, which makes them convenient models for mechanistic studies of post-transcriptional regulation of endogenous gene expression. We observed a rapid and significant decrease in cyclin protein, but not of mRNA levels after a knock-down (KD) of IMP3, suggesting that the regulation of cyclins expression by IMP3 occurred on post-transcriptional level. As the possibility of increased protein degradation of the cyclins in IMP3 KD cells was excluded by earlier studies in my laboratory, the main working hypothesis of my project was an involvement of translational repression as the principal regulatory mechanism.

RBPs can bind their target mRNAs co-transcriptionally in the nucleus and regulate their localization and translation, as it has been shown for IMP1 and Bruno [Singer RH, 2003 ; Snee M et al., 2008]. Previous study performed in my group has shown that IMP3 with a help of nucleocytoplasmic protein HNRNP M can enter the nucleus and bind the newly synthesized cyclin transcripts [Rivera Vargas, Boudoukha et al. 2014]. Therefore, a KD of IMP3 could stop the nuclear export of the cyclins mRNAs. However, we found that the nuclear export did not depend on IMP3, as the mRNAs of the cyclins were exported to the cytoplasm and associated with polysomes in the cytoplasm even when IMP3 was depleted. However, in IMP3 KD cells the cyclins mRNAs have preferentially perinuclear localization. It should be noted that such localization can be a consequence of translational inhibition of these transcripts by RNA-induced silencing complex (RISC), which is enriched in the perinuclear regions of mammalian cells [Stalder L et al., 2013].

IMP3 compete with RISC complex for cyclin D1 and D3 regulation.

We hypothesized that the decrease of the cyclins protein expression, observed in the absence of IMP3, could be caused by miRISC action. Minimal mammalian miRISC is composed of two key factors : GW182 protein that serves as a molecular platform and binds a multitude of silencing effectors ; and AGO2 protein with associated miRNA, which directs the silencing complex to the complementary target mRNA. In the majority of published studies, the action of miRISC was attributed to inhibition of translational initiation, or to a degradation of the target mRNA [Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Wakiyama et al., 2007; Wang et al., 2006]. However, miRISC can also regulate protein translation at post-initiation steps, promoting co-translational peptide degradation [Nottrott S et al., 2006], premature termination of translation by ribosomal drop-off [Petersen CP et al., 2006] and impaired elongation [Gu S et al., 2009]. Therefore, the molecular mechanisms of miR-mediated repression of protein expression remain extremely varied, depending on specific mRNA targets, miRNAs, and experimental models.

We found that the decrease of the cyclins D1, D3 after IMP-3 KD could be reversed by a KD of two key components of RISC complex: GW182 and AGO2. In all cases, inactivation of the RNAi machinery leads to a re-expression of the cyclins even in the absence of IMP-3. IMP3 partners PTBP1 and NF90/ILF3 act in the same way. These key findings led us to the conclusion that IMP3 and its protein partners, especially NF90/ILF3 and PTBP1, can protect their mRNA targets from RISC-dependent repression in human cancer cells.

Identification of functional regions within 3' UTRs of the cyclins, where a competition between IMP-3 and RISC complexes occurs.

The study of interaction between IMPs and their targets is very complex due to the absence of a unique target sequence. Furthermore, mRNA secondary structure also appears to play an important role in the target recognition by IMPs [Nielsen FC et al., 2002]. In this case, binding is based on the identification of the mRNA structures rather than on nucleotide sequence recognition. A large number of miRNAs are predicted to target cyclins D1 and D3, but are not confirmed experimentally. Therefore, we decided to experimentally map the functional sites within the cyclins 3' UTRs, where the IMP-3 binding competes with the RISC complex. Using the RNA-protein biotin pull-down technique, we identified regions bound by RISC complex components (GW182 and AGO2) and IMP3. Among them, we identified functional regions, where the binding efficiency of RISC complex components increased after

IMP3 KD, or the regions where the competition occurs. Then, we showed in vivo, that transfection of these RNA fragments, but not of the adjacent flanking regions, into IMP3 KD cells led to a partial or complete « rescue » of the cyclins expression. These findings further confirmed our hypothesis that IMP3 complexes protect the 3'UTRs of the cyclins mRNAs from GW182/AGO2 dependent translational repression.

Characterization of IGF2BP-containing cytoplasmic complexes.

IGF2BP/IMP proteins are mostly expressed in the cytoplasm, where they bind their target mRNAs and form cytoplasmic ribonucleoprotein complexes (mRNPs), dispersed preferentially around the nucleus [Nielsen et al., 2002]. These mRNP granules regulate translation, stability and transport of associated mRNAs. Unlike other well-known granules, like neuronal hStaufen and/or fragile X mental retardation protein granules, processing bodies (P-bodies), and stress granules [Jønson et al., 2007], the IMP granules lack eIF4E, eIF4G, and 60S ribosomal subunits, and contain CBP80 and exon-junction complex factors, suggesting that the associated mRNAs have never been translated.

IGF2BPs start controlling their target mRNAs co-transcriptionally in the nucleus and modulate the distribution of associated transcripts between translational and decay machineries or prevent microRNA attack by recruiting regulated transcripts in cytoplasmic mRNPs. Thus, Vg1 RBP/Vera (human IMP3 homologue) localizes Vg1 mRNA to the vegetal cortex of Xenophus oocyte [Kress et al., 2004]. ZBP1 (chicken homolog of IMP1) promotes localization of beta-actin mRNA to the extremities of growing neuronal cells [Oleynikov Y et Singer SH, 2003]. During transport, ZBP1 prevents premature translation of beta-actin mRNA, inhibiting its translational initiation. 'Caging' MYC and MDR1 in mRNP complexes, IGF2BP1 protects them from endonucleolytic cleavage and degradation [Lemm I and Ross J, 2002; Sparanese D and Lee CH, 2007]. IGF2BP1 also enhance BTRC1 expression by protecting its transcript from microRNA-mediated degradation [Elcheva et al., 2009]. A similar mechanism of forming IMP1/3-containing granules and stabilizing HMGA2 mRNA by protecting it against let-7-dependent degradation was proposed for IMP3 [Jønson et al., 2014]. It is interesting to note that these IMP3-containing granules lack translational initiation factors (eIF4E), RISC complex (Ago 1-4, miRNAs) and decay machinery (DCP1a) components. Besides HMGA2, IMP1/3 granules protect a variety of other miRNAs targets, making them inaccessible to RISC [Jønson et al., 2014].

The review of recent literature suggests that IGF2BPs participate in a network of complex RNA-protein and protein-protein interactions, suggesting a vast number of various IGF2BP-

containing regulatory mRNPs. This observation is in agreement with the post-transcriptional RNA operon model proposed by Keene and Tenenbaum (2002), according to which any given RNA contains multiple binding sites for various RNA-binding proteins. A different set and functions of trans-acting RBPs, bound to specific sequences on a given mRNA, determines its different cellular fate. Therefore, mRNAs containing similar cis-elements can be regulated jointly in the composition of such posttranscriptional regulons [Jønson et al., 2011].

According to our results, IMP3 co-interact with multiple RBPs in human cancer cells to positively regulate cyclin D1 and D3 translation, by protecting these mRNAs from GW182/AGO2/miRNA-dependent repression This regulatory mechanism, specific to IMP3 and not to its family members IMP1 and 2, remains fully reversible and very dynamic, as it does not change mRNA stability and does not involve de novo mRNA transcription. Two of IMP3 protein partners, PTBP1 and ILF3/NF90, have a strong impact on cyclin protein expression levels. The roles of these IMP3 partners could be elucidated by recent studies, which suggest that ILF3/NF90 regulates both mRNA translation and stability via cooperative or antagonistic interaction with miRISC action [Shim J et al., 2002 ; Xu YH et al., 2000; Xu YH et al., 2003]. PTBP1 binds to the 3′ UTR of mRNAs, modulating miRNA-mediating gene regulation [Bart Engels et al., 2012]. To conclude, in the course of my PhD thesis I have characterized a novel RNP regulatory complex, consisting of RNA-binding proteins IMP3, ILF3/NF90 and PTBP1. This complex binds to the mRNAs of cyclins D1 and D3 and protects these mRNAs from GW182/AGO2/miRNA dependent translational repression, thus ensuring continuous proliferation of human cancer cells.

PERSPECTIVES

1. IMP3 does not have a clearly defined binding sequence within its target RNAs, and it is hard to predict its binding sites. Our goal was to map the exact sites within the cyclins 3' UTRs, where the IMP3 binding competes with the RISC complex. We identified the functional fragments of 250 nt, where IMP3 binding competes with miRISC. In perspective, we can continue to cut these fragments in order to get an IMP3 pattern and compare it with the results of other methods (PAR-CLIP and others, bioinformatic approaches). By analyzing predicted miRNAs sites located near experimentally determined IMP3 target sequences, we can hypothesize the « hot spots » of translational regulation within the 3' UTRs of the target mRNAs, and then validate their functional significance by luciferase assays combined with mutations of miRNA sites and putative target sites of IMP3 complexes.

2. Our results indicate that IMP3 partners ILF3/NF90 and PTBP1 are important for CCND1 and CCND3 expression. However, NF90 is an RNA-dependent partner of IMP3, and could bind the mRNAs either as part of IMP3 complex, or independently, for examples after the RNA structure was changed by IMP3-PTBP1 binding. When the target sequence for IMP3 complexes within the 3' UTRs of the cyclins is characterized, it will be possible to elucidate these molecular events, their order and their relative importance for the competition with GW182.

3. It will be interesting to see how IMP3 physically interacts with its targets, as it has been shown for ZBP1 (IGF2BP1/IMP1 homolog), which binds to and regulates β-Actin mRNA. Especially, which domains of IMP3 and its partners are essential for initial mRNA binding and which ones are important for IMP3-mRNA complex stabilization in vivo. Structural analyses of subsequent protein–RNA co-crystals will be required for this approach.

4. Since 2007, there were an 'explosion' of reports, observing upregulation of IMP3 expression in various malignancies, correlating with tumor aggressiveness and metastasis. However, a pro-oncogenic role of IGF2BPs remains controversial due to the lack of 'classical' in vivo studies, involving transgenic mouse models. Using these models, we can analyze the role of IMP3 in cancer and validate in vivo a number of in vitro studies of its multiple mRNA targets regulation, among which are oncogenic factors (IGF2, MYC and others).

5. We have shown that IMP3 and RISC complexes can compete for cyclin D1 and D3 3`UTRs regulation. IMP3 can regulate cyclin 3`UTR length via alternative cleavage and

polyadenylation, resulting in a loss of miRISC binding sites. To test this hypothesis, we can perform analysis of alternative cleavage and polyadenylation of cyclin D1 and D3 mRNAs. IMP3 or another protein in its absence can regulate a polyA tail length. PolyA tail length positively correlates with miRISC association with mRNA via PABP. And to this case, it will be interesting to use several methods for measuring the length of the cyclin mRNA poly(A) tail.

6. In the long term, it will be interesting to explore the presence of secondary structures within 3' UTRs of the cyclins mRNAs, and to identify all clusters of motifs consisting of cytoplasmic polyadenylation elements (CPEs), AU-rich elements and another elements, such as the IMP3 complex target sites. Our work has clearly shown that these mRNAs are a convenient model for studies of post-transcriptional regulation of gene expression. A full « post-transcriptional interactome » of the 3' UTRs of the cyclins can serve as a blueprint of future studies in the filed.

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université PARIS-SACLAY

ÉCOLE DOCTORALE
Signalisation et réseaux intégratifs en biologie (BIOSIGNE)

Titre : Le complexe IMP3 protège ses ARNm cibles de la répression traductionnelle dépendante de Argonaute/GW182/miRNA.

Mots clés : IMP3, miRISC, les ARNm de cyclines.

Résumé : IMP-3 est difficilement détectable dans des tissus adultes normaux, mais il est surexprimée dans les nombreux cancers, où il a été rapporté comme un marqueur d'agressivité et de la croissance rapide tumorale, ainsi que d'un pronostic défavorable pour les patients. Dans notre recherche, nous avons utilisé un lignée cellulaire RD de rhabdomyosarcome (RMS), ou IMPs étaient décrits comme des protéines régulatrices de l'ARN de IGF-2. Nous avons essayé d'élucider le mécanisme par lequel IMP3 régule l'expression des cyclines D1 et D3, contribuant ainsi à la compréhension des processus oncogéniques dans RMS.
Nous avons montré que IMP3 régule l'expression des cycline D1 et D3 d'une manière significative in vivo. Nous avons également démontré, qu'en absence de IMP3, les ARNm des cyclines sont exportés vers le cytoplasme et associés avec les polyribosomes, mais ne sont pas traduits. En outre, l'inhibition d`IMP3 n'a pas d'influence à la stabilité des ARNm des cyclines.
Nous démontrons que, dans des cellules cancéreuses humaines, IMP3 interagit avec plusieurs protéines liant l'ARN, ce qui suggère l'existence d'une complexe régulatrice sur les 3'UTR des cyclines D1 et D3.
Nos résultats montrent que l'inhibition de deux protéines clés de RNA-induced silencing complex (Ago2 et TNRC6), délivrent les niveaux d'expression des cyclines D1 et D3, qui ont été considérablement diminué en abscence d'IMP3 ou de ses partenaires protéiques. Par conséquent, les complexes d`IMP3 et RISC peuvent concourir pour la régulation des ARNm des cyclines. Nous avons également identifié des miARNs, qui peuvent être impliqué dans ce processus et régions fonctionnelles dans les 3 'UTR des cyclines, où se passe la competition entre IMP-3 et RISC complexes.
Nos résultats sont compatibles avec l'existence de IMP3 - contenant complexe multiprotéique, qui est associé à 3'UTRs des cyclines et régule leur traduction en les protégeant contre la répression traductionnelle par miRISC.

Title : IMP3 complex protects its target mRNAs from Argonaute/GW182/miRNA dependent translational repression.

Keywords : IMP3, miRISC, cyclin mRNA.

Abstract : IMP-3 is hardly detectable in normal adult tissues, but is overexpressed in many cancers, where it has been reported as a marker of tumor aggressiveness, rapid growth, and bad prognosis for patients. In our research we utilized a rhabdomyosarcoma (RMS) cell line RD, where IMPs were first described as IGF-2 mRNA regulating proteins. We tried to elucidate the mechanism by which IMP3 regulates cyclins D1 and D3 expression, thereby contributing to the understanding of oncogenic processes in RMS.
We have shown that IMP3 regulates the expression of cyclin D1 and D3 in a significant manner in vivo. We also demonstrated, that in the absence of IMP3, the mRNAs of the cyclins are exported to the cytoplasm and associated with polyribosomes, but not translated. Moreover, IMP3 inhibition does not influence a stability of cyclin mRNAs.
We demonstrate, that in human cancer cells, IMP3 interacts with multiple RNA-binding proteins, suggesting the existence of regulatory RNP complex on the 3'UTR of the cyclin D1 and D3.
Our results show that an inhibition of two key proteins of RNA-induced silencing complex
(Ago2 and TNRC6), rescue the cyclin D1 and D3 protein expression levels, which were significantly decreased in the absence of IMP3 or its protein partners. Therefore, IMP3 and RISC complexes can compete for cyclin mRNAs regulation. We also identified a miRNAs, that can be involved in this process and functional regions within 3' UTRs of the cyclins, where a competition between IMP-3 and RISC complexes occurs.
Our results are consistent with the existence of IMP3 - containing multiprotein complex, which is associated with 3'UTRs of the cyclins and regulates their translation by protecting them from miRISC-dependent translational repression.

La synthese de la these en francais.

Introduction.

Les protéines se liant à l'ARN de la famille IMP 1, 2, et 3 (IGF2BP / IMP1-3) appartiennent à une famille conservée des protéines 'onco-foetales'. Leurs principales fonctions sont de réguler le transport, la stabilité et / ou la traduction des plusieurs ARNm. IMP sont fortement exprimés lors de l'embryogenèse et sont souvent régulés à la hausse dans un certain nombre de cancers. Selon plusieurs études, ces protéines 'onco-foetales' contrôlent la prolifération cellulaire, la différenciation, la migration, la polarisation et d`autres nombreux processus cellulaires. IMP3 est difficilement détectable dans les tissus adultes normaux, mais il est surexprimé dans de nombreux cancers, où il a été décrit comme un marqueur de l'agressivité tumorale, de la croissance rapide, et du mauvais pronostic aux patients. Des études antérieures effectuées par notre groupe ont montré que IMP3 lie l'ARNm des cyclines D1, D3 et régule positivement l'expression des cyclines sur le niveau de la protéine. Ce phénomène a été observé dans plusieurs lignées cancéreuses humaines. Dans cette étude, nous montrons que, en l'absence de IMP3, les ARNm des cyclines sont exportés vers le cytoplasme et associés avec polyribosomes, mais ne sont pas traduits. IMP3 inhibition n'a aucune influence sur la stabilité des ARNm des cyclines, et l'expression des cyclines peut être complètement inversée par une inactivation simultanée de GW182, une protéine essentielle pour la répression de la traduction par le complexe RISC. De plus, nous avons montré que dans les cellules cancéreuses humaines, IMP3 interagit avec des protéines liant l'ARN ILF3/NF90 et PTBP1, qui co-régulent l'expression des cyclines. Prises dans leur ensemble, nos résultats sont cohérents avec l'existence du complexe multiprotéique contenant IMP3, NF90 et PTBP1, qui se lie à des régions spécifiques des 3'UTRs des cyclines et les protège de la répression traductionnelle miRNA-dépendante.

IMP3 régule l`expression des cyclines D1 et D3 au niveau post-transcriptionnel.

Protéines se liant l'ARN dans les cellules s'associent à leurs ARNm cibles, résultant en formation des complexes de ribonucléoprotéine (mRNP). La composition de ces complexes détermine le destin cellulaire des ARNm: la localisation, la traduction et la dégradation. IMP forme des associations protéines-ARNm stables qui peuvent prévenir la dégradation prématurée et la traduction promiscuitée des transcriptes spécifiques, comme cela a été

montré pour CD44, MYC, PTEN ou BTRC [Lemm I et J Ross, 2002; Kobel M et al., 2007; Stohr N et al 2012. Vikesaa J et al., 2006; Noubissi FK et al., 2006]. En réglant ces ARNm, IMP3 a été reporté comme un promoteur de la progression tumorale et des métastases dans un certain nombre des tumeurs malignes.

Nous avons précédemment montré que IMP3 régule l'expression de la cycline D1 et D3 a la manière significative in vivo. Durée de la vie des cyclines est généralement courte avec de deux à quatre fois demi-vies plus courtes que leurs ARNm messagers, ce qui les rend les modèles pratiques pour des études mécanistiques de la régulation post-transcriptionnelle de l'expression des gènes. Par conséquent, mon projet de thèse vise à identifier les mécanismes moléculaires de la régulation de l'expression des cyclines par IMP3. Après une knock-down (KD) d`IMP3, nous avons observé une diminution rapide et significative des cyclines au niveau des protéines, mais pas au niveaux d'ARNm, ce qui suggère que la régulation de l'expression des cyclines par IMP3 se produit au niveau post-transcriptionnel. Comme la possibilité d'une forte dégradation des proteines des cyclines dans les cellules IMP3 KD a été exclue par des études antérieures dans mon laboratoire, la principale hypothèse du mon projet était une implication de la répression traductionnelle comme le principal mécanisme de la régulation des cyclines par IMP3.

Les protéines se liant à l'ARN peuvent interagir avec leurs ARNm cibles a la manière co-transcriptionnel dans le noyau et réguler leur localisation et la traduction, comme il a été montré pour IMP1 et Bruno [Chanteur RH, 2003; Snee M et al., 2008]. Une étude précédente réalisée dans mon groupe a montré que IMP3 avec une aide de la protéine nucleocytoplamique HnRNP M peut entrer dans le noyau et se lier les transcrites des cyclines nouvellement synthétisées [Rivera Vargas, Boudoukha et al. 2014]. Nous avons adressé une question si les ARNm des cyclines ont un problème avec leur export nucléaire en absence d`IMP3. Cependant, nous avons constaté que l'export nucléaire des cyclines ne dépend pas d`IMP3, et que leur ARNm ont été exportés dans le cytoplasme et sont associés avec des polysomes même en absence d`IMP3. Cependant, dans les cellules IMP3 KD les ARNm des cyclines semble avoir localisation préférentiellement périnucléaire. Il est à noter qu'une telle localisation peut être une conséquence de l'inhibition de la traduction de ces transcrits par un complexe de silençage induit par ARN (RISC), qui est enrichi dans les régions périnucléaires des cellules de mammifères (Stalder L et al., 2013).

La interaction entre les protéines se liant à l'ARN et les miARN sur la 3` UTRs de l`ARN.

La région 3'UTR de l'ARNm, en jouant un rôle important dans la régulation post-transcriptionnelle de l'expression génique, contrôle cette expression par une diaphonie entre les composants structurels de l'ARNm (cis-éléments) et les facteurs agissant en trans (miRNA ou les protéines se liant à l'ARN). Ces molécules peuvent se lier aux sites communs ou distincts de la cible d'ARNm, de manière compétitive. Les protéines se liant à l'ARN et miARNs peuvent réguler la traduction et la stabilité de l'ARNm à la manière positive ou négative. En même temps, ils peuvent coopérer ou être antagonistes dans leur actions, et le résultat final dépend de la collective de nombreuses circonstances, telles que leur niveau d'expression, l'affinité pour des sites de liaison, et la localisation dans la cellule, qui peuvent être régulés par de différentes conditions physiologiques.

La coopération entre les miARN et les protéines se liant à l'ARN sur la région 3`UTR de l'ARNm.

Les protéines se liant à l'ARN participent activement à la régulation miARN-dépendante de l'expression génique. Par exemple, les protéines Pumilio (PUM1 et PUM2) collaborent avec plusieurs miARNs et agissent comme des guides qui interviennent dans l'ouverture de la structure secondaire d'ARNm, permettant des interactions entre miARN et leurs sites de liaison. Une étude comparative des sites cibles de Pumilio au sein de l'ARNm, en utilisant RIP-CHIP et PAR-CLIP dans les cellules humaines, a montré un enrichissement pour les sites de reconnaissance par miARN dans les séquences voisines des sites de liaison de Pumilio, ce qui suggère une interaction généralisée des protéines Pumilio humains avec les systèmes miRNA-dépendantes [Galgano A. et al., 2008].

Kedde et collaborateurs [Kedde M et al., 2010] ont mis en évidence dans la lignée cellulaire de cancer du sein MCF7, que Pumilio favorise les effets inhibiteurs de miR-221/222 sur le p27Kip1 ARNm par l'ouverture de la structure secondaire de la 3'UTR de p27Kip1, permettant l'accès de miR-221/222 à son site de liaison.

Un modèle élégant a été proposé pour une action coopérative de HuR et let-7 sur la région 3 'UTR de c-Myc [HH Kim et al., 2009]. HuR peut lier la 3 'UTR de l'ARNm de c-Myc dans une position proche de let-7 ce qui réduit l'expression de c-Myc. L'activité de let-7 est

dépendante de Hur, et l'activité de Hur dépend de let-7. Les auteurs ont découvert que la liaison HuR modifie la conformation locale de l'ARNm et favorise l'association de let-7 avec l'ARNm de c-Myc, en démasquant site de reconnaissance par let-7.

Les protéines se liant à l'ARN peuvent interagir directement avec AGO et recruter le RISC.

Le complexe RISC peut avoir une interaction physique directe avec les protéines se liant à l'ARN. Dans une première étude, Caudy et ses collègues ont démontré que le complexe RISC chez la drosophile contient des protéines qui associent avec des éléments riches en AU, comme la protéine FXR1 [Caudy AA et al., 2002]. La réduction de FXR1 dans des cellules S2 de Drosophile a entraîné le silencing d'ARNi moins efficace. D'autres travaux ont confirmé que FXR1 associés avec Ago2 dans des cellules mammifères [Jin P et al., 2004]. La découverte d'une interaction fonctionnelle entre FXR1 et Ago2 conduit à des suggestions qui soit les protéines se liant a l'ARN sont ciblées sur leurs ARNm par l'ARNi complexe [Caudy AA et al., 2002], soit FXR1 scanne pour le G quartet, précédemment rapporté comme un site de reconnaissance par FXR1, et recrute le RISC ensuite.

Les protéines se liant à l'ARN contre les fonction des miARN sur la 3`UTR.

L'activité de miARN peut également être inhibée par les protéines se liant à l'ARN, par une mécanisme de la compétition pour les sites de liaison commune sur la région 3'UTR. DND1, une protéine de liaison à l'ARN qui assure la médiation la viabilité des cellules germinales et supprime la formation de tumeurs germinales [Kedde M et al., 2007], a été montrée pour interagir avec les extrémités 3 'UTR de l'ARNm p27Kip1 et de l'ARNm LATS2. DND1 lie ces ARNm et interfère avec la fonction des miARNs, en aidant à maintenir les niveaux des protéines p27Kip1 et LATS2 (les suppresseurs de tumeurs) dans une lignée cellulaire de tumeur des cellules germinales et dans le carcinome épidermoïde, inhibant ainsi la tumorigenèse. Généralement, il existe deux mécanismes d'action de DND1: liaison de DND1 peut entraîner la formation de structure secondaire sur ARNm qui rend inaccessibles les sites de reconnaissance des miARN, ou encore, la liaison de DND1 peut changer une localisation subcellulaire de l'ARNm, en la rendant inaccessible au complexe RISC.

Un autre exemple d'interactions antagonistes intéressant entre les protéines se liant à l'ARN et miARN a été démontrée par Bhattacharyya SN et Filipowicz W. Ils ont effectué une étude élégante qui démontre que la répression par miARN est une processus réversible. La

régulation négative du transporteur cationique d'acides aminés 1 (CAT-1) ARNm par miR-122 a été inhibée par le stress, qui en meme temps a favorisé l'élévation du niveau de Hur cellulaire, principalement nucléaire, et permet son entrée dans les P-bodies cytoplasmiques (structures de dégradation d'ARNm), la liaison à la séquence spécifique riche en AU de CAT-1 et à la relocalisation de CAT1 ARNm à partir des P-bodies à polysomes [SN Bhattacharyya et al., 2006]. Récemment, les mêmes auteurs ont démontré un mécanisme d'action possible de HuR sur la région 3 'UTR de CAT-1. Comme les AREs Hur sont positionnés à une distance considérable des sites des miRNA, HuR favorise le soulagement de la répression par miARN à distance par un processus impliquant probablement HuR oligomérisation [Kundu P et al., 2012].

Par conséquent, les protéines se liant à l'ARN peuvent interagir directement avec le complexe RISC (RNA-induced silencing complex), soit stabilisant la liaison de celui-ci avec des ARNm cibles, soit antagonisant les fonctions des miRNA.

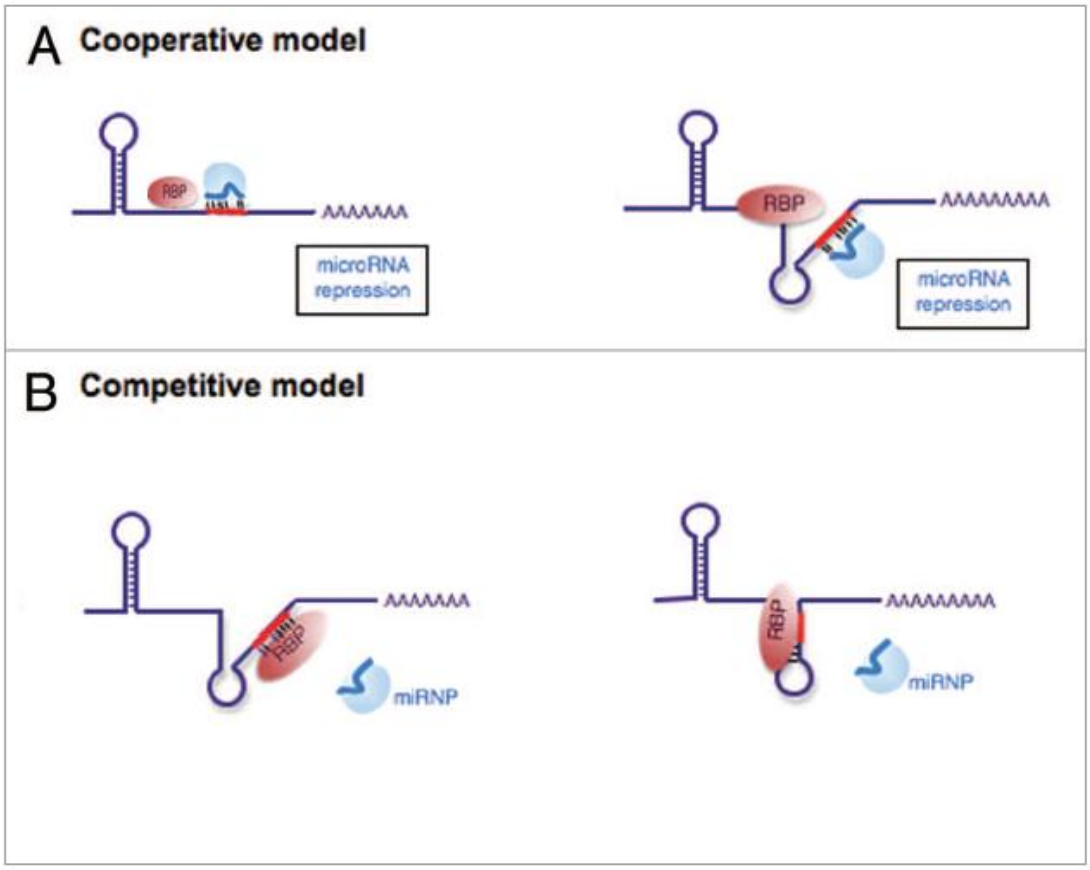

Fig.1. Intéractions des miRNA avec les protéines se liant à l'ARN dans la régulation de la même ARNm [de Ciafrè SA et al., 2013, avec les modifications].

A) La Coopération: coté gauche: les protéines se liant à l'ARN peuvent favoriser la fonction des miARN en ouvrant une structure secondaire, ce qui permet l'interaction entre miRNA et ce site cible au sein de la région 3`UTR. Sur la coté droite: les protéines se liant à l'ARN peuvent recruter les composants du complexe RISC vers la région 3`UTR et stabiliser leur liaison par l'interaction directe avec des composants du RISC. B) La compétition: les protéines se liant à l'ARN peuvent interférer avec la fonction des miARN via la concurrence pour le même site de liaison. Dans le contexte des sites qui ne se chevauchent les protéines se liant à l'ARN peuvent changer la structure locale de l'ARNm et prevenir la liaison avec miARN.

IMP3 rivalise avec un complexe RISC pour la régulation des cyclines D1 et D3.

Nous avons supposé que la répression des cyclines, observée en l'absence d`IMP3, peut être causé par l'action du miRISC. Le miRISC minimale des mammifère est composé de deux facteurs clés: de la protéine GW182/TNRC6 qui servent comme un plate-forme moléculaires et lient une multitude d'effecteurs de silençage, et de la protéine Ago2 associés avec miARN. Dans la majorité des études publiées, miRISC a été détecté comme un inhibiteur de la traduction au niveau de initiation et comme un promoteur de la dégradation d`ARNm cible [Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Wakiyama et al., 2007; Wang et al., 2006]. Cependant, miRISC peut également réguler la traduction au niveau de post-initiation, en promouvant la dégradation co-traductionnelle des peptides [Nottrott S et al., 2006], la terminaison prématurée de la traduction par un drop-off ribosomique [Petersen CP et al., 2006] et l`élongation altérée [Gu S et al., 2009]. Nous avons constaté que la diminution des cyclines D1, D3 après IMP-3 KD peut être inversée par KD de deux éléments clés du complexe RISC: GW182 et Ago2. Dans tous les cas, l'inactivation de la machinerie d'ARNi conduit à une ré-expression des cyclines, même en absence d'IMP-3. Les partenaires d`IMP3, PTBP1 et NF90, peuvent agir de la même manière. Ces résultats clés nous ont amenés à conclure que IMP3 et ses partenaires protéiques, en particulier NF90 et PTBP1, peuvent protéger leurs ARNm cibles de la répression RISC-dépendante dans les cellules cancéreuses humaines.

L`identification des régions fonctionnelles dans les 3 'UTRs des cyclines, où une concurrence entre les complexes IMP-3 et RISC a lieu.

L'étude de l'interaction entre IMP3 et ses cibles est très complexe en raison de l'absence d'une séquence unique de liasion par IMP3. En outre, la structure secondaire de l'ARNm semble également jouer un rôle important dans la reconnaissance de ses cibles par IMP3 [FC Nielsen et al., 2002]. Dans ce cas, la liaison est basée sur l'identification des structures d'ARNm plutôt que sur la séquence nucléotidique d`ARNm. Un grand nombre de miARN sont prédits pour cibler les cyclines D1 et D3, mais ils ne sont pas confirmés expérimentalement. Par conséquent, nous avons décidé de chercher expérimentalement les sites fonctionnels dans les 3 'UTR des cyclines, où a lieu la concurrence de liaison entre IMP-3 et le complexe RISC. En utilisant la technique ARN-protéine biotine pulldown, nous avons identifié les régions liées par des composants de complexe RISC (GW182 et Ago2) et IMP3. Parmi eux, nous avons identifié des régions fonctionnelles, où l'efficacité de liaison des composants du complexe RISC augmente après IMP3 KD et la concurrence se produit. Ensuite, nous avons montré in vivo, que la transfection de ces fragments d'ARN, mais pas des régions adjacentes dans les cellules IMP3 KD, provoque un «rescue» partiel ou complet de l'expression des cyclines. Ces résultats confirment notre hypothèse selon laquelle complexes IMP3 protège les 3'UTRs des cyclines de la répression traductionnelle, GW182 / Ago2 dépendente.

La caractérisation des IGF2BP-complexes cytoplasmiques.

IGF2BP / IMP protéines sont principalement exprimés dans le cytoplasme, où ils se lient à leurs ARNm cibles en formant des complexes de ribonucléoprotéine (mRNP cytoplasmiques), qui sont préférentiellement dispersées autour du noyau [Nielsen et al., 2002]. Ces granules mRNP régulent la traduction, la stabilité et le transport des ARNm associés. Contrairement à d'autres granules bien connus, comme neuronale hStaufen et / ou fragiles X granules de protéines de l'arriération mentale, les P-bodies, et les granules de stress [Jonson et al., 2007], les granulés IMP manquent eIF4E, eIF4G et 60S sous-unités ribosomiques, et contiennent des facteurs d`exon-jonction complexes et CBP80, ce qui suggère que les ARNm associés ont jamais été traduits.

IGF2BPs commencent à contrôler leurs ARNm cibles co-transcriptionnellement dans le noyau en modulant la distribution de transcrits associés entre les machineries de la traduction

et de la dégradation et peuvent prévenir l`attaque des microARN en recrutant des transcrits régulés dans mRNP cytoplasmiques. Ainsi, Vg1 RBP / Vera (homologue de IMP3 chez l`Homme) regule le transport d`ARNm Vg1 pour le cortex végétal de ovocyte de Xenophus [Kress et al., 2004]. ZBP1 (un homologue de IMP1 chez le poulet) favorise la localisation de bêta-actine aux extrémités de la culture de cellules neuronales [Oleynikov Y et Singer SH, 2003]. Pendant le transport, ZBP1 empêche la traduction prématurée de la bêta-actine, en inhibant son initiation traductionelle. En liant MYC et MDR1 dans les complexes de mRNP, IGF2BP1 les protège du clivage endonucléolytique et de la dégradation [Lemm I et J Ross, 2002; Sparanese D et Lee CH, 2007]. IGF2BP1 améliore également l'expression de BTRC1 en protégeant son transcript de la dégradation microARN dépendante [Elcheva et al., 2009]. Un mécanisme similaire qui se compose de la formation des granules IMP1/3 et de la stabilisation de l`ARNm HMGA2 en le protégeant contre la dégradation let-7-dépendante, a été proposé pour IMP3 [Jonson et al., 2014]. Il est intéressant de noter que ces granules contenant IMP3 manquent facteurs d'initiation de la traduction (eIF4E), complexe RISC (AGO 1-4, miARN) et les composants de la machine de la dégradation (DCP1a). Outre HMGA2, les granules IMP1/3 protégent une variété d'autres ARN cibles de miARN, les rendant inaccessibles au RISC [Jonson et al., 2014].

La revue de la littérature récente suggère que IGF2BPs participent à un réseau d'interactions ARN-protéine et protéine-protéine, ce qui suggère un grand nombre de diverses complexes réglementaires mRNP contenant IMPs. Cette observation est en accord avec le modèle d`ARN opéron post-transcriptionnel proposé par Keene et Tenenbaum (2002), selon lequel toute ARN contient multiples sites de liaison pour diverses protéines liant l'ARN. Un ensemble et fonctions differen**tes** des protéines se liant à l`ARN, liés à des séquences spécifiques sur un ARNm, détermine leur différent destin cellulaire. Par conséquent, les ARNm contenant cis-éléments similaires peuvent être réglés conjointement dans la composition de ces régulons post-transcriptionnels [Jonson et al., 2011].

Selon nos résultats, IMP3 co-interagi avec des multiples protéines se liant à l`ARN dans les cellules cancéreuses humaines et régule positivement la traduction des cyclines D1 et D3, en protégeant leur ARNm du GW182 / Ago2 / miARN-dépendante répression. Ce mécanisme réglementaire, spécifique à IMP3 mais pas aux autres membres de sa famille, IMP1 et IMP2, reste entièrement réversible et très dynamique, car il ne se compose pas du changement de la stabilité de l'ARNm et ne comporte pas de novo transcription de l'ARNm. Deux partenaires protéiques d`IMP3, PTBP1 et ILF3 / NF90, ont un fort impact sur les niveaux d'expression de

protéines des cyclines. Les rôles de ces partenaires d`IMP3 pourraient être élucidés par des études récentes qui suggèrent que ILF3 / NF90 régule à la fois la traduction et la stabilité des ARNm grâce à l'interaction coopérative ou antagoniste avec l'action du miRISC [Shim J et al., 2002; Xu YH et al., 2000; Xu YH et al., 2003]. PTBP1 se lie à 3'UTR de l'ARNm, en promotant la modulation miARN-dépéndantes de la régulation des gènes [Bart Engels et al., 2012]. Pour conclure, dans le cadre de ma thèse de doctorat, j'ai caractérisé un nouveau complexe régulateur RNP, constitué des protéines liant l'ARN comme IMP3, ILF3 / NF90 et PTBP1. Ce complexe se lie à l'ARNm des cyclines D1 et D3 et protège ces ARNm de la répression traductionnelle GW182 / Ago2 / miRNA dépendante, assurant la prolifération continue des cellules cancéreuses humaines.

Basé sur les résultats de notre étude, nous avons proposé un modèle selon lequel IMP-3 et ses partenaires se lient les ARNm de CCND1 et D3 dans les cellules cancéreuses humaines, et cet événement rend ces transcrits compétents pour la traduction en les protégeant contre la répression traductionnelle par miRISC action.

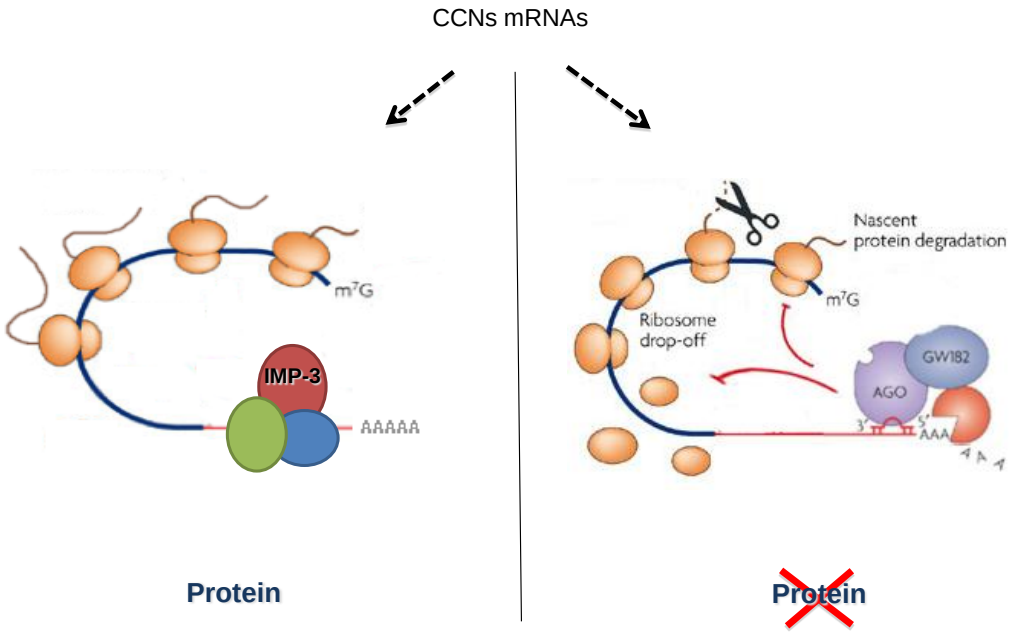

Fig .2. Le modèle proposé pour la régulation de l'éxpression des cyclines par le mécanisme de la compétition entre les complexes IMP3 et RISC.

Les perspectives.

1. Le IMP3 n'a pas de séquence de liaison pour ses ARN cibles clairement définies, et il est difficile de prédire ses sites de liaison. Notre objectif était de « cartographier » les sites exacts des 3 'UTR des cyclines, où il y a une concurrence pour la liaison entre les complexes RISC et IMP3. Nous avons identifié les fragments fonctionnels de 250 nt, où IMP3 concurre avec miRISC. En perspective, nous pouvons continuer à réduire ces fragments afin d'obtenir un site de liasion d'IMP3 et la comparer avec les résultats d'autres méthodes (PAR-CLIP et d'autres, les approches bioinformatiques). En analysant les sites des miARN prévus près des séquences d'IMP3 expérimentalement déterminée**s**, nous pouvons identifier les «points chauds» de la régulation de la traduction des ARNm cibles, et valider leur signification fonctionnelle par des essais de luciférase combinés avec des mutations des sites des miRNA et/ou des putatifs sites d'IMP3 complexe.

2. Nos résultats indiquent que les partenaires d'IMP3, ILF3/NF90 et PTBP1, sont importants pour l'expression des CCND1 et CCND3. Cependant, NF90 est un partenaire l'ARN-dépendant de IMP3, et pourrait lier les ARNm soit dans la composition du complexe IMP3, ou indépendamment, après la modification de la structure de l'ARN par la liaison IMP3-PTBP1. Lorsque la séquence de la liasion pour le complexe IMP3 sera caractérisée, il sera possible d'élucider ces événements moléculaires, leur ordre et leur importance relative pour la concurrence avec RISC.

3. Il sera intéressant de voir comment IMP3 interagit physiquement avec ses ARNm, comme il a été montré pour ZBP1 (IGF2BP1 / homologue de IMP1), qui se lie à l'ARNm de β-actine. Surtout, il est intéressant de déterminer les domaines de IMP3 et ses partenaires qui sont essentiels pour la liaison de l'ARNm et ceux qui sont importants pour la stabilisation de complexe IMP3-ARNm in vivo. Les analyses structurales de protéines-ARN et des études de co-crystallisation seront nécessaires pour cette approche.

4. Depuis 2007, il y avait une «explosion» des rapports, qui ont observé une régulation positive de l'expression d'IMP3 dans diverses tumeurs malignes, qui est en corrélation positive avec l'agressivité des tumeur et les métastases. Cependant, un rôle pro-oncogénique de IGF2BPs reste controversé en raison de l'absence des études «classiques» in vivo, impliquant des modèles des souris transgéniques. En utilisant ces modèles, nous pouvons analyser le rôle des IMP3 dans le cancer et de valider in vivo un certain nombre d'études in

vitro de régulations de ses multiples ARNm cibles, parmi lesquels sont des facteurs oncogènes (IGF2, MYC et autres).

5. Nous avons montré que les complexes IMP3 et RISC peuvent concourir pour la régulation des 3`UTRs des cyclines D1 et D3. IMP3 peut régler la longueur de la région 3`UTR des cyclines par un clivage alternatif et une polyadénylation, résultant en une perte de sites de liaison avec miRISC. Pour tester cette hypothèse, on peut effectuer des analyses de clivage et polyadénylation alternative des ARNm des cyclines D1 et D3 en presence ou en absence d'IMP3. En absence d'IMP3, une autre protéine peut réguler une longueur de la queue polyA. La longueur de la queue PolyA est positivement corrélée avec l'association de miRISC avec l'ARNm via PABP. Et pour ce cas, il sera intéressant d'utiliser plusieurs méthodes pour mesurer la longueur de la queue poly (A) des cyclines.

6. Dans le long terme, il sera intéressant d'explorer la présence des structures secondaires dans les 3 'UTR des cyclines, et d'identifier toutes les grappes de motifs constitués d'éléments de polyadénylation cytoplasmique (CPE), des éléments riches en AU et autres éléments , tels que les sites de liasion du complexe IMP3. Notre travail a clairement montré que ces ARNm présentent un modèle idéal pour les études de régulation post-transcriptionnelle de l'expression génique. Un plein «interactome post-transcriptionnelle» de 3 'UTR des cyclines peut servir comme un modèle des futures études dans le domaine.

Nous aimerions trouver d'autres ARNm, régulé**s** par IMP3 par le mécanisme que nous avons décrit pour CCND1 et CCND3. Des études antérieures dans mon laboratoire ont montré que ce mécanisme dépend de la import nucléaire d'IMP3, et à cela, il serait intéressant d'identifier tous les ARNm enrichi**s** en IMP-3 complexes des fractions nucléaires purifiée**s**, et d'analyser leur régulation par IMP-3. CCND1 et D3 peuvent servir comme les controles positifs dans cette étude.

Par la suite, il sera intéressant d'identifier les éléments structurels au sein des ARNm, qui déterminent leur régulation par une concurrence entre IMP3 et RISC. En utilisant les techniques que j'ai mis en route au cours de mon travail de thèse, il sera possible de caractériser les protéines se liant à l'ARN et les miARN impliqués dans ce mécanisme de régulation. Ainsi, un effet général du phénomène que nous avons décrit pour l'ARNm de CCND1 et D3 peut être évalué dans le contexte de la prolifération cellulaire et l'oncogenèse.